Characterization of neurometabolic 2-hydroxyglutaric acidurias and discovery of D-2-hydroxyglutaric aciduria type II

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Martijn Kranendijk

2012

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VRIJE UNIVERSITEIT

Characterization of neurometabolic 2-hydroxyglutaric acidurias and discovery of D-2-hydroxyglutaric aciduria type II

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
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in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
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door

Martijn Kranendijk

geboren te Delft

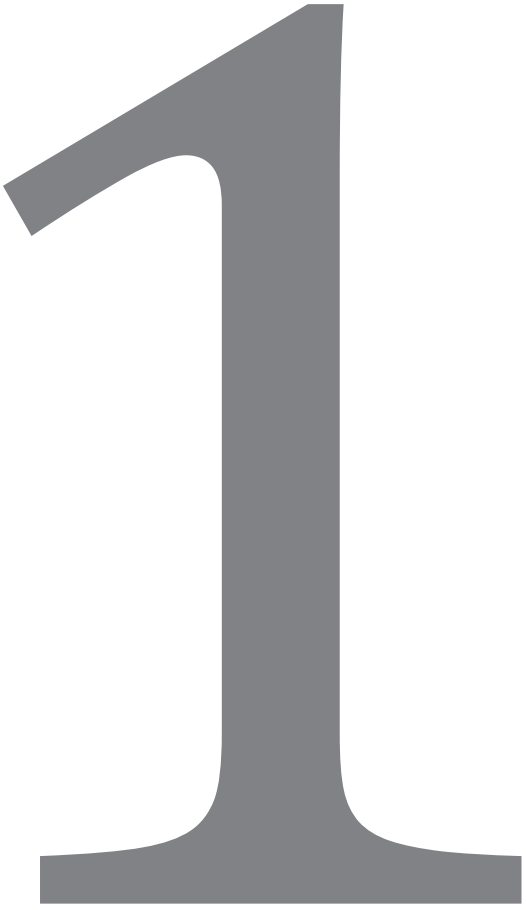
promotor:	prof.dr.ir. C.A.J.M. Jakobs
copromotoren:	dr. E.A. Struijs
	dr. G.S. Salomons

I am indebted to the patients and their caretakers
whose support made this research possible.

Your contributions have led to new knowledge and
expanded interest, and may lead eventually to
effective therapeutics for these diseases.

Content

Objectives and outline of the thesis



The work presented here is the continuation of ongoing research initiated in 1980 with the discovery of two neurometabolic disorders D-2-hydroxyglutaric aciduria (D-2-HGA) and L-2-hydroxyglutaric aciduria (L-2-HGA) [1,2]. Severe accumulation of D-2-hydroxyglutarate (D-2-HG) and L-2-hydroxyglutarate (L-2-HG) in body fluids are the biochemical hallmarks of these disorders respectively. A third disorder characterized by moderate accumulation of both D-2-HG and L-2-HG is recognized and denoted "combined D- and L-2-hydroxyglutaric aciduria" (D,L-2-HGA) [3]. All these disorders manifest in young children and express neurological impairments.

All the above mentioned 2-hydroxyglutaric acidurias (2-HGA) can be detected by urine organic acid analysis, a technique available in many metabolic laboratories. Chiral differentiation of 2-hydroxyglutarate is needed to establish the biochemical diagnosis, and this specialized technique is available in our metabolic laboratory. As a consequence our laboratory serves as a biochemical and genetic reference center for 2-HGA, allowing us to study and stratify these rare metabolic disorders.

At the start of this thesis in 2008, it was known that mutations in the *L2HGDH* gene cause L-2-HGA [4,5]. However, a functional assay to determine L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) activity in patient materials was not available. Mutations in the *D2HGDH* gene were found to be causative for D-2-HGA in eight patients [6-8], but the genetic defect in many other D-2-HGA patients remained undisclosed. At that time, mild and severe phenotypes were recognized in D-2-HGA [9,10], but correlations of the observed phenotype with genotype or metabolite levels in body fluids were not demonstrated. The etiology of D,L-2-HGA, which is characterized with a severe phenotype with a shortened lifespan, has been poorly understood [3].

Over the last two decades biological matrices, DNA, cells and clinical questionnaires were collected to establish a sustainable and highly valued fundament of biological specimens and clinical data which was an absolute requirement to accomplish this thesis. In a multidisciplinary setting of metabolic, enzymatic, genetic and clinical studies subsequent objectives were formulated:

1. Development of an L-2-HGDH enzyme assay in cultured cells derived from L-2-HGA patients.
2. Exploration of phenotype-genotype-metabolite correlations in D-2-HGA.
3. Identification of the defect in D-2-HGA patients without mutations in the *D2HGDH* gene.
4. Exploration of therapeutic interventions in D-2-HGA and L-2-HGA.

An L-2-HGDH enzyme assay in cell lysates, based on the conversion of stable isotope labeled L-2-HG to stable isotope labeled 2-ketoglutarate (2-KG), was developed (**Chapter 2**). Impaired L-2-HGDH activity was detected in all L-2-HGA patients investigated, confirming the diagnosis and affirming unambiguously the link between genetic and metabolic observations in these patients.

Evidence for genetic heterogeneity in D-2-HGA was established with the differentiation of at least two distinct groups with unique genetic, enzymatic and biochemical properties (**Chapter 3**). In a cohort of 50 patients, 24 individuals carried two mutations in the *D2HGDH* gene and had impaired D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) activity. We denoted this form of D-2-HGA as type I.

The discovery of recurrent mutations in *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) in neoplastic disorders revealed a gain-of-function mechanism in mutated-IDH enzymes producing vast amounts of D-2-HG [11,12]. This finding led us to the discovery of *de novo* heterozygous germline mutations in *IDH2* in D-2-HGA patients who had no mutations in *D2HGDH* and normal D-2-HGDH activity (**Chapter 4**). These affected patients were denoted D-2-HGA type II.

Subsequently, a functional assay was developed to determine the novel IDH2-mutant gain-of-function enzyme activity in D-2-HGA type II patient's lymphoblast lysates (**Chapter 5**). This assay confirmed the diagnoses and served as a study model to explore therapeutic interventions.

Genetic, enzymatic and metabolic definition of D-2-HGA type I and type II defined 95% of the total D-2-HGA population, leaving several individual cases unidentified. Clinical data collected with an international questionnaire from patients affected with either D-2-HGA type I or type II established the phenotype of these patients which were included in a *Review* encompassing D-2-HGA, L-2-HGA and D,L-2-HGA (**Chapter 6**).

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Development and implementation of a novel assay for L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) in cell lysates: L-2-HGDH deficiency in 15 patients with L-2-hydroxyglutaric aciduria

M. Kranendijk, G.S. Salomons, K.M. Gibson, C. Aktuglu-Zeybek, S. Bekri, E. Christensen, J. Clarke, A. Hahn, S.H. Korman, V. Mejaski-Bosnjak, A. Superti-Furga, C. Vianey-Saban, M.S. van der Knaap, C. Jakobs, E.A. Struys

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ORIGINAL ARTICLE

Development and implementation of a novel assay for L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) in cell lysates: L-2-HGDH deficiency in 15 patients with L-2-hydroxyglutaric aciduria

M. Kranendijk · G. S. Salomons · K. M. Gibson · C. Aktuglu-Zeybek · S. Bekri · E. Christensen · J. Clarke · A. Hahn · S. H. Korman · V. Mejaski-Bosnjak · A. Superti-Furga · C. Vianey-Saban · M. S. van der Knaap · C. Jakobs · E. A. Struys

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Summary L-2-hydroxyglutaric aciduria (L-2-HGA) is a rare inherited autosomal recessive neurometabolic disorder caused by mutations in the gene encoding L-2-hydroxyglutarate dehydrogenase. An assay to evaluate L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) activity in fibroblast, lymphoblast and/or lymphocyte lysates has hitherto been unavailable. We developed an L-2-HGDH enzyme assay in cell lysates based on the conversion of stable-isotope-labelled L-2-hydroxyglutarate to 2-ketoglutarate, which is converted into L-glutamate in situ. The formation of stable isotope labelled L-glutamate is therefore a direct measure of L-2-HGDH activity, and this product is detected by liquid chromatography-tandem mass spectrometry. A deficiency of L-2-HGDH activity was detected in cell lysates from 15 out of 15 L-2-HGA patients. Therefore,

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References to electronic databases: L-2-Hydroxyglutaric aciduria: OMIM 236792. L-2-Hydroxyglutarate dehydrogenase: EC 1.1.99.2. *L2HGDH* gene: NM_024884.2. L-Malate dehydrogenase: EC 1.1.1.37. D-2-Hydroxyglutarate dehydrogenase: EC 1.1.99.-. L-Glutamate dehydrogenase: EC 1.4.1.3.

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this specific assay confirmed the diagnosis unambiguously affirming the relationship between molecular and biochemical observations. Residual activity was detected in cells derived from one L-2-HGA patient. The L-2-HGDH assay will be valuable for examining in vitro riboflavin/FAD therapy to rescue L-2-HGDH activity.

Abbreviations

CSF	cerebrospinal fluid
FBS	fetal bovine serum
Fmoc	1-(9-fluorenyl)methylchloroformate
[$^2H_4$]2-KG	2-keto[3,3,4,4-$^2H_4$]glutaric acid
2-HG	2-hydroxyglutarate
GC-MS	gas chromatography-mass spectrometry
HBSS	Hanks balanced salt solution
INT	iodonitrotetrazolium chloride
2-KG	2-ketoglutarate
L-[$^{13}C_5$]glu	L-[$^{13}C_5$]glutamic acid
L-[$^2H_4$]2-HG	L-[3,3,4,4-$^2H_4$]2-hydroxyglutaric acid
L-[$^2H_4$]glu	L-[3,3,4,4-$^2H_4$]glutamic acid
L-2-HG	L-2-hydroxyglutarate
L-2-HGA	L-2-hydroxyglutaric aciduria
L-2-HGDH	L-2-hydroxyglutarate dehydrogenase
L2HGDH	L-2-hydroxyglutarate dehydrogenase gene
LC-MS/MS	liquid chromatography-tandem mass spectrometry
L-GluDH	L-glutamate dehydrogenase
P/S	penicillin/streptomycin

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Introduction

L-2-Hydroxyglutaric aciduria (L-2-HGA, OMIM 236792) was first described in 1980 in a patient with mental and motor developmental delay and growth deficiency (Duran et al. 1980). The clinical phenotype of patients with L-2-HGA is characterized by progressive ataxia and mental deficiency and on MRI a subcortical leukoencephalopathy and cerebellar atrophy, classifying this disease as a true neurological disorder with characteristic/diagnostic MRI findings (Barbot et al. 1997; Barth et al. 1993; Steenweg et al. 2009; Topcu et al. 2005). Moreover, brain tumours of variable nature have been reported in L-2-HGA (Aghili et al. 2009; Haliloglu et al. 2008; Larnaout et al. 2007). The enormous increase in 2-hydroxyglutarate (2-HG) excretion, as detected by urinary organic acid profiling, is a strong diagnostic clue. Subsequent chiral specification and quantification of 2-HG by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) reveals the isolated increase of the L-isomer, which is also increased in cerebrospinal fluid (CSF) and plasma, with a CSF to plasma ratio >1 (Duran et al. 1980; Gibson et al. 1993; Struys et al. 2004). Modest elevations of L-lysine in CSF and plasma appears to be a secondary phenomenon in L-2-HGA patients (Barth et al. 1992, 1993), and it has been hypothesized that depleted 2-ketoglutarate (2-KG) impairs the conversion of L-lysine into saccharopine (Kamoun et al. 2002).

Recent studies have uncovered the underlying genetic basis of L-2-HGA: mutations were found in the gene *L2HGDH* (NM_024884.2, located on 14q22.1) encoding L-2-hydroxyglutarate dehydrogenase (L-2-HGDH, EC 1.1.99.2) (Rzem et al. 2004; Topcu et al. 2004). This FAD-dependent membrane-bound enzyme is responsible for the conversion of L-2-hydroxyglutarate (L-2-HG) into 2-KG. L-2-HG originates from the non-specific conversion of 2-KG by mitochondrial L-malate dehydrogenase (EC 1.1.1.37) (Rzem et al. 2007). By analysis of isotopomer distributions in L-2-HG, citrate and 2-KG following supplementation of stable-isotope-labelled glucose or L-glutamate to cell culture media of L-2-HGA fibroblasts, Struys and co-workers verified that 2-KG is the direct precursor of L-2-HG (Struys et al. 2007). Since the metabolic function of L-2-HG is unknown, L-2-HGDH might be regarded as an enzyme of metabolic repair (Rzem et al. 2007), potentially preventing the loss of important carbon moieties. Several investigators have suggested that the pathophysiology of L-2-HGA involves chronic cerebral intoxication related to supraphysiological levels of

L-2-HG (da Silva et al. 2003; Latini et al. 2003). However, altered cerebral 2-KG pools may also be a factor.

The identification of the *L2HGDH* gene has expanded the diagnostic approaches for this disease. L-2-HGA is genetically homogeneous, as illustrated by the consistent findings of increased L-2-HG in body fluids and homozygous or compound heterozygous mutations or deletions in DNA from L-2-HGA patients (Sass et al. 2008; Topcu et al. 2004; Vilarinho et al. 2005).

However, in the diagnostic laboratory the triad of metabolite-enzyme-DNA was not possible, because a functional enzyme assay in readily accessible human cells such as fibroblasts, lymphoblasts or lymphocytes was previously unavailable (Rzem et al. 2004; Van Schaftingen et al. 2009). The enzyme assays described by Rzem and co-workers, both radioisotopic and spectrophotometric, lack the sensitivity to measure L-2-HGDH activities in the cell types of interest. In the current study our aim was to develop a specific and sensitive enzyme assay to determine L-2-HGDH activity. Recently, we reported a stable-isotope-labelled enzyme assay for the determination of D-2-hydroxyglutarate dehydrogenase (D-2-HGDH, EC 1.1.99.-) activity in human fibroblasts and lymphoblasts, based upon the conversion of purified D-[$^2H_4$]2-hydroxyglutarate via [$^2H_4$]2-ketoglutarate into L-[$^2H_4$]glutamate (Wickenhagen et al. 2009). Here, we apply this approach to the assessment of L-2-HGDH activities in cultured cells from controls and patients, and correlate the outcome with molecular and metabolic findings.

Materials and methods

Subjects

Fibroblast cell lines (n=11), lymphoblast cell lines (n=5) and lymphocyte pellets (n=3) derived from 15 patients affected with L-2-HGA, diagnosed on the basis of increased excretion of L-2-HG and mutations in the *L2HGDH* gene, were used. Urinary L-2-HG concentration determination was performed according to the protocols described by Gibson and by Struys (Gibson et al. 1993; Struys et al. 2004). Mutations/deletions in the 10 exons encompassing the *L2HGDH* gene were determined by DNA/cDNA sequencing analysis and/or multiplex ligation-dependent probe amplification (MLPA). Nine fibroblast and four lymphoblast cell lines, and four lymphocytes pellets, all derived from individuals not affected with L-2-HGA, served as controls.

Chemicals and reagents

Stable-isotope-labelled L-[$^{13}C_5$]glutamic acid (L-[$^{13}C_5$]glu), Hepes, iodonitrotetrazolium chloride (INT), 1-(9-fluorenyl)methyl chloroformate (Fmoc), and L-glutamate dehydrogenase enzyme (L-GluDH, EC 1.4.1.3) were purchased from Sigma-Aldrich (St Louis, MO, USA). Stable-isotope-labelled 2-keto[3,3,4,4-$^2H_4$]glutaric acid ([$^2H_4$]2-KG) was obtained from Euriso-Top (Gif sur Yvette, France). L-[3,3,4,4-$^2H_4$]2-hydroxyglutaric acid (L-[$^2H_4$]2-HG) was prepared in-house by reducing [$^2H_4$]2-KG to racemic D/L-[$^2H_4$]2-HG (Gibson et al. 1993). Subsequently, L-[$^2H_4$]2-HG was isolated by chiral HPLC using a Phenomenex (D)-penicillamine column (Wickenhagen et al. 2009). To determine substrate purity, traces of [$^2H_4$]2-KG contamination in the purified L-[$^2H_4$]2-HG substrate were converted to L-[3,3,4,4-$^2H_4$]glutamic acid (L-[$^2H_4$]glu) by the addition of commercially available L-GluDH, and the resulting L-[$^2H_4$]glu was measured by LC-MS/MS as described below.

Culture media Ham F10, RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin (P/S), Hanks balanced salt solution (HBSS), and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). All other solvents and chemicals were of analytical grade.

Cell culture and isolation

Fibroblasts were cultured in 75 cm^2 flasks with Ham F10 medium containing 1% P/S and 10% FBS until near confluency. Cells were trypsinized, washed with HBSS and stored as a dry cell pellet at −80°C prior to analysis. Lymphoblasts were grown in 50 ml RPMI 1640 medium supplemented with 1% P/S and 10% FBS until a sufficient cell suspension was formed. A cell pellet was obtained by centrifugation of 15 ml cell suspension (6 min at 340 *g*), washed and stored as described above. Lymphocytes were isolated from EDTA whole blood within 48 h after sampling using Accuspin System-Histopaque-1077 tubes (Sigma-Aldrich) and stored at −80°C prior to analysis.

L-2-Hydroxyglutarate dehydrogenase assay

Cell pellets were cooled on ice and resuspended in 400 µl of freshly prepared buffer containing 20 mmol/L Hepes pH 7.0, 0.86 mmol/L $MgCl_2$, and 1.5 mmol/L INT. The cells were disrupted by ultrasonication using a Bandalin Sonopuls mini 20 titanium 1.5 mm probe for 10 s at 90% power while cooled on ice. The lysate was mixed gently, 75 µl was transferred in duplicate into two 1.5 ml glass vials and 5 µl 2.34 mmol/L L-[$^2H_4$]

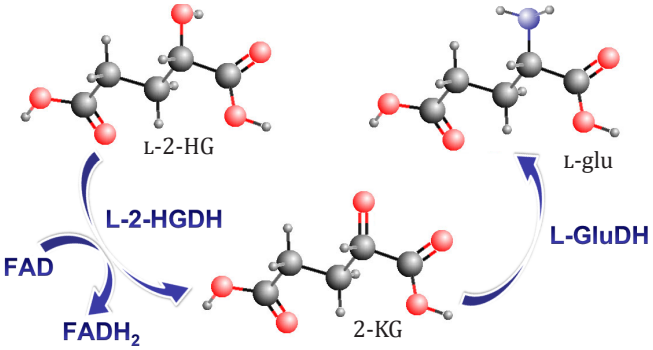

Fig. 1 L-2-Hydroxyglutarate dehydrogenase (L-2-HGDH) oxidizes L-2-hydroxyglutarate (L-2-HG) to 2-ketoglutarate (2-KG), using FAD as a cofactor. The second step is the reductive amination of 2-KG to L-glutamate (L-glu) by L-glutamate dehydrogenase (L-GluDH)

2-HG substrate was added to initiate the assay, after which the incubation was performed for 30 min at 37°C. L-[$^2H_4$]2-HG is oxidized by L-2-HGDH to form [$^2H_4$]2-KG, which is immediately converted in situ to L-[$^2H_4$]glu by the reductive amination reaction of endogenous L-GluDH (Fig. 1). The formation of L-[$^2H_4$]glu is thus a direct measure for L-2-HGDH activity.

The lysate was cooled on ice immediately after incubation, loaded onto a Millipore Microcon Ultracel YM-10 kDa filter, and centrifuged for 30 min at 10 000 *g* at 4°C for protein removal. The filtrate was used for LC-MS/MS analysis. The enzyme assay was optimized and investigated for buffer composition, pH, temperature, incubation time, and substrate and protein dependences.

Since L-GluDH enzyme is used in the second step of the assay, a control assay was performed to determine its activity to prevent false-negatives. The procedure is identical to the preceding assay, except for the replacement of the substrate by 5 µl 0.1 mmol/L [$^2H_4$] 2-KG. This second assay also provides a control analysis for enzyme viability in the cell lysate.

LC-MS/MS analysis

Samples were prepared by transferring 40 µl of filtrate into a 300 µl glass vial followed by the addition of internal standard solution (for fibroblast lysates 10 µl of 0.5 µmol/L L-[$^{13}C_5$]glu and for lymphoblast and lymphocyte lysates 10 µl of 5 µmol/L L-[$^{13}C_5$]glu), 125 µl of buffer (125 mmol/L boric acid pH=10), and 125 µl reagent (1.5 mg/ml Fmoc in acetone). Solutions were mixed at room temperature to convert L-[$^2H_4$]glu, L-[$^{13}C_5$]glu, and endogenous L-glutamate (L-glu) to their Fmoc derivatives.

Liquid chromatography was performed using a Waters Acquity HPLC equipped with a Waters XTerra RP_{18} column operating at room temperature (5 µm 3.9×150 mm+5 µm 3.9×20 mm pre-column) using a flow rate of 0.8 ml/min and an injection volume of 10 µl. A linear gradient was applied over 4 min starting from 100% mobile phase A (10% acetonitrile, 120 mg/L aqueous ammonium formate) to 100% mobile phase B (60% acetonitrile, 120 mg/L aqueous ammonium formate) followed by 4 min 100% mobile phase B.

The Applied Biosystems 4000 Q TRAP tandem mass spectrometer was equipped with a TurboIonSpray source operating in negative ionization mode at 550°C. The LC column was connected splitlessly to the tandem MS system. Other system settings were: CUR=15 psi, CAD=5, IS=−3000 V, GS1=30 psi, GS2=20 psi, ihe = ON, DP=−40 V, EP=−10 V, CE=−15 V, and CXP=−13 V. Fmoc derivatives were detected in multiple-reaction-monitoring mode (MRM) using transitions for L-glu −368.1→−172.1 *m/z*, L-[$^2H_4$]glu −372.1→−176.1 *m/z*, and L-[$^{13}C_5$]glu −373.1→−177.1 *m/z* with 100 ms dwell time. Data

Table 1 Kinetic parameters L-2-HGDH assay

Cell type/tissue	V_{max} (pmol/h per mg protein, n=1)	K_m (µmol/L, n=1)	Intra-precision CV (%)	Inter-precision CV (%)
Fibroblasts	564	112	8.2 (n=7)	22 (n=5)
Lymphoblasts	5152	107	2.1 (n=5)	5.4 (n=5)
Rat liver[a]	6000	150	–	–

[a] Rzem et al. (2004).

acquisition and processing was performed with Applied Biosystems Analyst 1.4.2 software.

Results

Assay optimization

L-2-HGDH activity was highest in 20 mmol/L Hepes pH 7.0 and 0.86 mmol/L $MgCl_2$. The exogenous electron-proton acceptor INT (1.5 mmol/L) increased V_{max} ~10-fold and K_m ~50-fold (data not shown), which was necessary to obtain sufficient sensitivity to identify activity in fibroblasts. During incubation, the activity was linear for 30 min, and was linear over a lysate protein concentration range of 0.1–0.6 mg/ml for fibroblasts and 0.3–1.5 mg/ml for lymphoblasts. Temperature in the range of 30–39°C did not significantly alter enzyme activity.

V_{max} and K_m values were calculated from Lineweaver—Burk plots of substrate saturation studies (Table 1). Lymphoblasts demonstrated a 10-fold higher V_{max} but comparable K_m values when compared with fibroblasts, i.e. 107 and 112 µmol/L, which was comparable with the previously published K_m (150 µmol/L) assessed in rat liver (Rzem et al. 2004). Intra-precision was ≤8.2% coefficient of variation (CV) and the inter-precision was ≤22% CV, which validates the assay for determination of enzyme deficiency in fibroblasts and lymphoblasts. There was sufficient L-2-HGDH activity in lymphocytes, which should facilitate the use of this cell type.

Determination of L-2-HGDH enzyme activity

Cells derived from all L-2-HGA subjects and controls were measured in duplicate. All cell lines from controls and L-2-HGA tested positive for the internal control L-glutamate dehydrogenase enzyme assay assuring the quality of the cell homogenates (data not shown). All L-2-HGA cell lysates had significant decreased L-2-HGDH activity compared with controls (Fig. 2). Fibroblasts derived from patient 11 showed residual activity of 51 pmol/h per mg protein, which was still 5 times lower than average control activity of 245± 70 pmol/h per mg protein (95% confidence interval (CI), Table 2). In all remaining subjects no residual L-2-HGDH activities were found in the cell lysates, i.e. <1% of the mean L-2-HGDH activity of controls. The urinary L-2-HG excretion in patients ranged from 671 to 3392 mmol/mol creatinine, which is at least 35-fold higher than controls (1.3–18.9 mmol/mol creatinine).

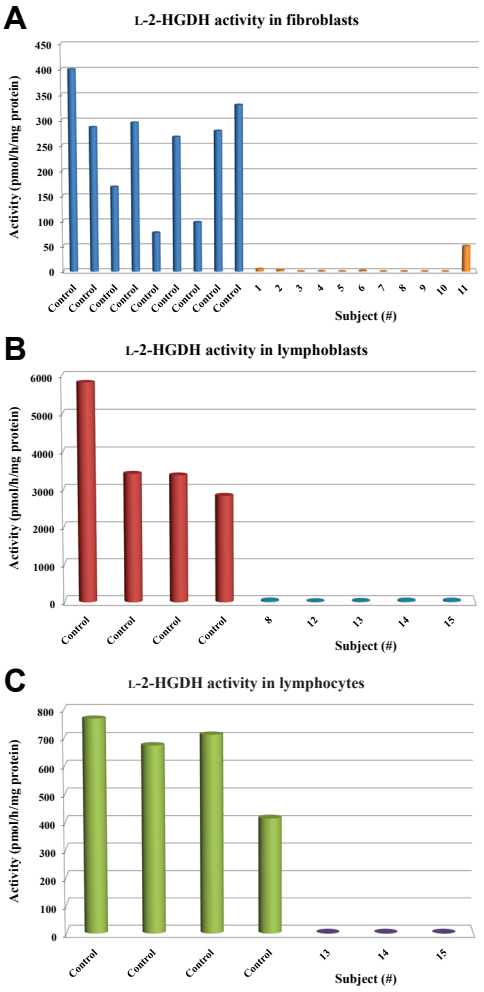

Fig. 2 L-2-HGDH enzyme activity in fibroblasts (**A**), lymphoblasts (**B**) and lymphocytes (**C**) revealing decreased/absent activity for L-2-HGA subjects compared with controls

Discussion

We present a novel L-2-HGDH enzyme assay suitable for use in fibroblasts, lymphoblasts and lymphocytes derived from L-2-HGA-affected individuals and controls. All L-2-HGA patients demonstrated a pronounced deficiency of L-2-HGDH activity, which correlates with the high levels of L-2-HG in body fluids (Table 2). This assay provides a functional link between the biochemical and molecular

Table 2 L-2-HGDH assay results and urinary L-2-HG concentrations

Subject	L-2-HGDH enzyme activity (pmol/h per mg protein ± CI (95%))			Urinary L-2-HG (mmol/mol creatine)
	Fibroblasts	Lymphoblasts	Lymphocytes	
1	5			951
2	3			1928
3	0			1732
4	0			2171
5	1			3392
6	2			1880
7	0			1702
8	0	31		2723
9	0			671
10	0			2214
11	51			2291
12		11		2657
13		18	0	2352
14		28	0	2138
15		26	0	1592
Control (*n*) min-max	245±70 (9) 78–400	3867±1305 (4) 2838–5825	642±154 (4) 414–769	1.3–18.9

features of this disorder. The possibility of applying this assay to lymphocytes makes this method a quick diagnostic tool circumventing laborious cell culturing. Our method can be readily applied to those circumstances in which there is uncertainty about the diagnosis; i.e. for genetic variants of unknown significance, or when no DNA studies are feasible. During the two-step assay the substrate L-[$^2H_4$]2-HG is converted by L-2-HGDH into [$^2H_4$]2-KG, which is subsequently converted into L-[$^2H_4$]glu by L-GluDH. Product L-[$^2H_4$]glu is quantified with LC-MS/MS, which is the measure for L-2-HGDH enzyme activity. A major advantage with our assay is the use of enantiomerically pure substrate L-[$^2H_4$]2-HG, which is mandatory since D-2-HGDH is present in cellular lysates and also generates the same product(s) as L-2-HGDH; any contribution of D-2-HGDH activity interferes with the detection of product L-[$^2H_4$]glu in the LC-MS/MS analysis.

There are currently no satisfactory treatment options for L-2-HGA patients. However, two cases were recently published in which oral supplementation of riboflavin (Yilmaz 2009) or FAD plus carnitine (Samuraki et al. 2008) were promising, with improved clinical signs for both. Since, L-2-HGDH is FAD-linked, we speculate that riboflavin and FAD may stabilize mutant L-2-HGDH in these patients. Unfortunately, no cells were available from these patients to determine the L-2-HGDH activity. Nevertheless, cells derived from all tested L-2-HGA patients but one showed essentially zero enzyme activity, but this does not preclude the possibility of stabilization of the L-2-HGDH enzyme in a tissue-specific manner.

Conversely, fibroblasts derived from patient 11 displayed a modest residual activity, i.e. 20% of the mean L-2-HGDH activity of controls. It is of note that this patient is compound heterozygous for two missense mutations: a relatively mild type of mutation. However, cells derived from three other L-2-HGA patients who were also compound heterozygous for two missense mutations displayed no residual L-2-HGDH activities. Patient 11 presented with mild psychomotor developmental delay, choreodystonia and behavioural problems, and MRI showed a leukodystrophy and basal ganglia abnormalities. The clinical presentation of patient 11, as well as the urinary L-2-HG concentration, is not distinguishable from the other 14 cases, despite the detected residual L-2-HGDH activity. However, the residual activity in patient 11 suggests that a trial with riboflavin/FAD may be efficacious. In the future, we want to evaluate the effect of riboflavin/FAD supplementation in vitro with fibroblasts and lymphoblasts derived from patient 11 and others to determine whether L-2-HGDH activity can be increased and thus intracellular L-2-HG concentrations can be decreased.

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Evidence for genetic heterogeneity in D-2-Hydroxyglutaric aciduria

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RESEARCH ARTICLE

Human Mutation

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Evidence for Genetic Heterogeneity in D-2-Hydroxyglutaric Aciduria

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ABSTRACT: We performed molecular, enzyme, and metabolic studies in 50 patients with D-2-hydroxyglutaric aciduria (D-2-HGA) who accumulated D-2-hydroxyglutarate (D-2-HG) in physiological fluids. Presumed pathogenic mutations were detected in 24 of 50 patients in the *D-2-hydroxyglutarate dehydrogenase* (*D2HGDH*) gene, which encodes D-2-hydroxyglutarate dehydrogenase (D-2-HGDH). Enzyme assay of D-2-HGDH confirmed that all patients with mutations had impaired enzyme activity, whereas patients with D-2-HGA whose enzyme activity was normal did not have mutations. Significantly lower D-2-HG concentrations in body fluids were observed in mutation-positive D-2-HGA patients than in mutation-negative patients. These results imply that multiple genetic loci may be associated with hyperexcretion of D-2-HG. Accordingly, we suggest a new classification: D-2-HGA Type I associates with D-2-HGDH deficiency, whereas idiopathic D-2-HGA manifests with normal D-2-HGDH activity and higher D-2-HG levels in body fluids compared with Type I patients. It remains possible that several classifications for idiopathic D-2-HGA patients with diverse genetic loci will be revealed in future studies.
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KEY WORDS: D-2-Hydroxyglutarate dehydrogenase; *D2HGDH*; D-2-HGA; D-2-HGDH; D-2-hydroxyglutaric aciduria

Additional Supporting Information may be found in the online version of this article.

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Introduction

D-2-Hydroxyglutaric aciduria (D-2-HGA; MIM# 600721) is a neurometabolic disorder with a wide clinical spectrum, ranging from asymptomatic to affected children with epilepsy, cardiomyopathy, hypotonia, facial dysmorphic features, and truncated lifespan [Korman et al., 2004; Struys et al., 2005a; van der Knaap et al., 1999a,b]. There is no phenotype–metabolite correlation with regard to D-2-HG levels. The description of mitochondrial D-2-hydroxyglutarate dehydrogenase (D-2-HGDH; MIM# 609186, EC 1.1.99.-) [Achouri et al., 2004] led to the identification of autosomal recessively inherited mutations in the human *D-2-hydroxyglutarate dehydrogenase* gene (*D2HGDH* gene, NM_152783.3, located on 2p25.3) that associate with increased D-2-HG in body fluids. Thus far, eight presumed pathogenic mutations in the *D2HGDH* gene have been described, two of which reside in intervening sequences and six within the coding region [Haliloglu et al., 2009; Misra et al., 2005; Struys et al., 2005a,b]. In line with the earlier noticed absence of a correlation between the biochemical and clinical phenotype, no genotype–phenotype correlation was observed, consistent with the absence of a phenotype–metabolite correlation [van der Knaap et al., 1999a,b]. This is highlighted by monozygotic D-2-HGA twins, compound heterozygote for *D2HGDH* mutations [Misra et al.,

2005], in which the first-born presented with multiple congenital anomalies, facial dysmorphia, and severe developmental delay, while the twin sister presented with only facial dysmorphia.

We have recently developed methodology for direct sequencing of the *D2HGDH* coding region and splice sites at the gDNA and mRNA levels, as well as Multiplex Ligation-dependent Probe Amplification (MLPA) techniques to examine deletions in the *D2HGDH* gene. We have now supplemented these molecular techniques with a sensitive functional enzyme assay employing stable isotope precursors to determine D-2-HGDH activity in cultured human cells, to confirm and differentiate mutation-positive and mutation-negative patients [Wickenhagen et al., 2009]. The current report summarizes our molecular, enzymatic, and biochemical characterizations of a large cohort of D-2-HGA patients, employing the investigative tools described above, and provide data indicating the involvement of multiple loci in D-2-HGA.

Subjects and Methods

Subjects

Fifty individuals with increased levels of D-2-HG in urine, plasma, and/or cerebrospinal fluid (CSF) were studied. Patients who excreted increased levels of L-2-HG were excluded. DNA was obtained from all subjects. Fibroblasts or lymphoblasts from 25 subjects were available for D-2-HGDH enzyme activity determination. The subjects emanated from 18 countries, including Argentina (2), Australia (1), Austria (1), Belgium (1), Canada (3), Denmark (2), France (1), Germany (2), Israel (5), Italy (3), Netherlands (7), Norway (1), Poland (3), Singapore (1), Sweden (1), Thailand (1), United Kingdom (5), and the United States (10).

Molecular Sequencing/MLPA

DNA was isolated from blood or cultured cells using the QIAamp blood kit (Qiagen, Chatsworth, CA). Sequence-specific primers were used to amplify all 10 exons and the adjacent splice sites of the *D2HGDH* gene for all patients (Supp. Table S1). RNA was isolated from cultured cells grown without and with cycloheximide to inhibit nonsense mediated decay using the SV Total RNA isolation kit (Promega, Madison, WI). Approximately 1 μg was used to synthesize cDNA with Reverse Transcriptase (RT) (Qiagen) according to manufacturer's instructions. Full-length cDNA was amplified using different primer sets (Supp. Table S1). The (RT-)PCRs were performed with HotStar Taq (Qiagen) (one cycle 95°C 15 min, 35 cycles of 45 sec 94°C, 1 min 58°C, 1 min 72°C followed by an extension step of 7 min at 72°C) using an PE Applied Biosystems (Bedford, MA) machine model 9700. Direct sequencing of all amplicons was performed on purified PCR products (Millipore vacufold, Bedford, MA) using the BigDye Terminator v3.1 cycle sequencing kit and an ABI 3130XL sequencer (PE Applied Biosystems). The electropherograms were analyzed with the Mutations Surveyor software package (SoftGenetics, State College, PA). DNA alterations were annotated according to the guidelines of Den Dunnen and Antonarakis (http://www.hgvs.org/mutnomen/) [den Dunnen and Antonarakis, 2000]. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, NM_152783.3, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Generic MLPA was performed to screen for genomic deletions and duplications in the *D2HGDH* gene using the MLPA-kit P107 v03 0107 (MRC-Holland). The analysis was performed in duplicate and the products analyzed using the ABI 3130XL genetic analyzer. Data analysis employed the GeneMarker MLPA system (Softgenetics).

Enzyme Assay

The D-2-HGDH enzyme assay employing cultured human cells has been described [Wickenhagen et al., 2009]. In brief, the assay utilized fibroblast or lymphoblasts clarified extracts, obtained by sonification at 4°C, and enantiomerically pure D-2-[3,3,4,4-$^2H_4$]hydroxyglutaric acid (D-2-[$^2H_4$]HG) as substrate. D-2-[$^2H_4$]HG is converted by D-2-HGDH to 2-keto[3,3,4,4-$^2H_4$]glutaric acid (2-[$^2H_4$]KG), a reactive intermediate subsequently converted to L-[3,3,4,4-$^2H_4$]glutamic acid (L-[$^2H_4$]glu) by endogenous L-glutamate dehydrogenase. The formation of the latter represents a measure of D-2-HGDH activity, and quantification is achieved by liquid chromatography-tandem mass spectrometry using L-[$^{13}C_5$]glutamic acid as internal standard. L-Glutamate dehydrogenase activity was determined to establish extract viability and as a control, using 2-[$^2H_4$]KG as substrate followed by measurement of L-[$^2H_4$]glu.

Metabolite Assays

Quantification of D- and L-2-HG was performed in physiological fluids using stable isotope dilution gas chromatography-mass spectrometry [Gibson et al., 1993] or stable isotope dilution liquid chromatography-tandem mass spectrometry [Struys et al., 2004a]. 2-Ketoglutarate (2-KG) was estimated retrospectively in GC-FID chromatograms of the urinary organic acid profile.

Results

Molecular Investigations

Homozygous, or compound heterozygous, mutations were detected in 24 of 50 D-2-HGA patients in the *D2HGDH* gene. Schematic representation of the gene depicts 29 mutations (21 novel; Fig. 1). Novel mutations included 7 that are considered pathogenic because these predict truncated proteins, including a splice donor site (c.853+2T>C), two exonic deletions (c.(?_30)_853+?del [exon1-6]; c.1141-?_(*220_?) [exon9-10]), two nonsense (c.505C>T, p.Gln169X; c.1200C>A, p.Tyr400X) and two frame shift (c.642delT, p.Arg215AspfsX25; c.1333_1334del, p.Thr445GlyfsX103) alterations. The remaining 14 mutations include missense mutations not detected in 210 control alleles, and altering conserved amino acids across phyla. This observation suggests pathogenicity.

For the remaining unrelated 26 D-2-HGA patients, no pathogenic *D2HGDH* alterations were detected. RT-PCR confirmed an absence of splice errors in 11 of 11 patients, and further confirmed bi-allelic expression in 9 cases for whom nondisease coding variants were detected. Thus, the molecular genetic alteration of D-2-HGA in >50% of our patients remains undefined.

Enzyme Assay

Reduced D-2-HGDH enzyme activity was detected in only 7 of the 25 D-2-HGA patients of whom cell lines were available (Table 1). All seven manifested homozygous or compound heterozygous mutations in the *D2HGDH* gene, which likely correlate with impaired D-2-HGDH enzyme function. Normal D-2-HGDH activity in parents of patient 7, both of whom harbor

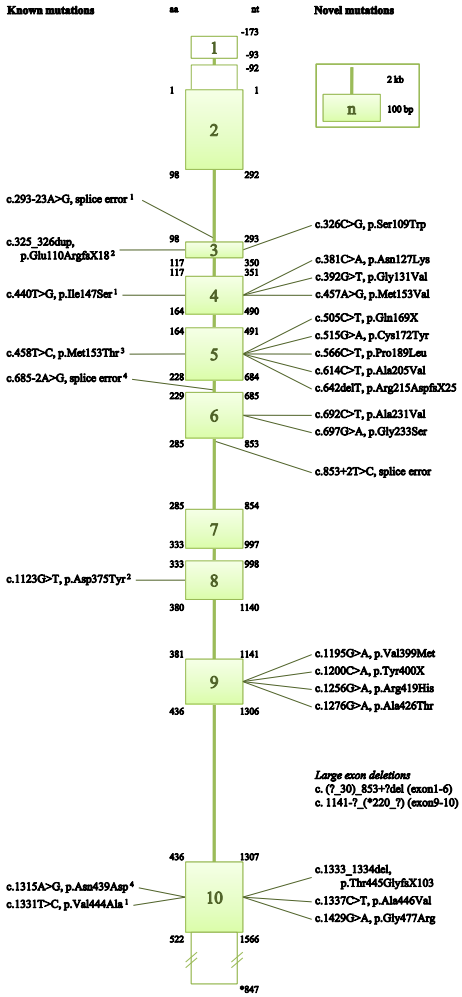

Figure 1. Schematic representation of the *D2HGDH* gene showing all currently known mutations [1] [Struys et al., 2005b] [2] [Misra et al., 2005] [3] [Haliloglu et al., 2009] [4] [Struys et al., 2005a]. Nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1. The initiation codon is codon 1. All sequence variants are described according to den Dunnen and Antonarakis (http://www.hgvs.org/mutnomen/) [den Dunnen and Antonarakis, 2000] and are checked by using the Mutalyzer program (NM_152783.3 (mRNA), NG_012012.1 (gDNA)) [Wildeman et al., 2008].

a heterozygous mutation, support an autosomal recessive inheritance pattern for D-2-HGDH deficiency (Table 1).

All remaining 18 D-2-HGA patients displayed D-2-HGDH enzyme activities within the control range (Table 1). Normal enzyme activities, in conjunction with an absence of *D2HGDH* gene mutations, support the concept of loci heterogeneity in this disorder.

Table 1. D-2-HGDH Enzyme Activity in 25 D-2-HGA Patients

		D-2-HGDH activity (pmol h^{-1} mg $protein^{-1}$)	
Subject (♯)	Mutation in *D2HGDH*	Fibroblasts	Lymphoblasts
1	c.[1256G>A(+)1256G>A]	39	
2	c.[685-2A>G(+)685-2A>G]	28	
3	c.[440T>G]+[293-23A>G]	41	
4	c.[515G>A(+)515G>A]	4	
5	c.[1200C>A(+)1276G>A]	0	
6	c.[642delT]+[853+2T>C]		21
7	c.[326C>G]+[457A>G]		2
8	Mutation absent	218	
9	Mutation absent	280	
10	Mutation absent	204	
11	Mutation absent	228	
12	Mutation absent	373	
13	Mutation absent	348	
14	Mutation absent	565	
15	Mutation absent	261	
16	Mutation absent	252	
17	Mutation absent	234	
18	Mutation absent	634	
19	Mutation absent	437	
20	Mutation absent	242	
21	Mutation absent	449	
22	Mutation absent		1,113
23	Mutation absent		1,061
24	Mutation absent		1,503
25	Mutation absent		570
Mother subject 7	c.[326C>G]+[=], not affected		633
Father subject 7	c.[457A>G]+[=], not affected		885
Control cell lines[a] ($n = 5$)		247–665	273–2,545

[a][Wickenhagen et al., 2009].

Metabolite Analysis

We next correlated D-2-HG levels in physiological fluids with the molecular genetic and enzyme data, which revealed a clear distinction between patient subgroups. Mutation-negative D-2-HGA patients displayed significantly higher levels of D-2-HG in urine and plasma (Fig. 2; $P < 0.01$). The trend in CSF was consistent but failed to achieve statistical significance, perhaps due to the lower sample number. Urinary 2-KG levels, estimated for 30 patients, were within normal limits (data not shown) [Blau et al., 2008]. However, mutation-negative D-2-HGA patients demonstrated slightly higher urinary 2-KG concentrations than mutation-positive patients (data not shown).

Discussion

We have identified more than 85 patients with D-2-HGA in the recent 15 years via detection of increased D-2-HG levels in urine, plasma, and/or CSF, and we have now been able to subgroup these patients using molecular, enzymatic, and metabolite profiling. Our data now reveal the involvement of at least a second locus in D-2-HGA. Accordingly, we suggest a new classification of the disease D-2-hydroxyglutaric aciduria. D-2-HGA Type I denotes a disorder associated with mutations in the *D2HGDH* gene and impaired D-2-HGDH enzyme activity. Conversely, idiopathic D-2-HGA denotes a disorder in which no mutations are present in the *D2HGDH* gene and associated with normal D-2-HGDH enzyme activity. Thus far, our experience indicates that idiopathic D-2-HGA patients excrete significantly higher levels of D-2-HG than levels observed in D-2-HGA Type I patients.

Analysis of D-2-HGDH activity is performed in crude homogenates of fibroblasts and lymphoblasts. Because D-2-HGDH is

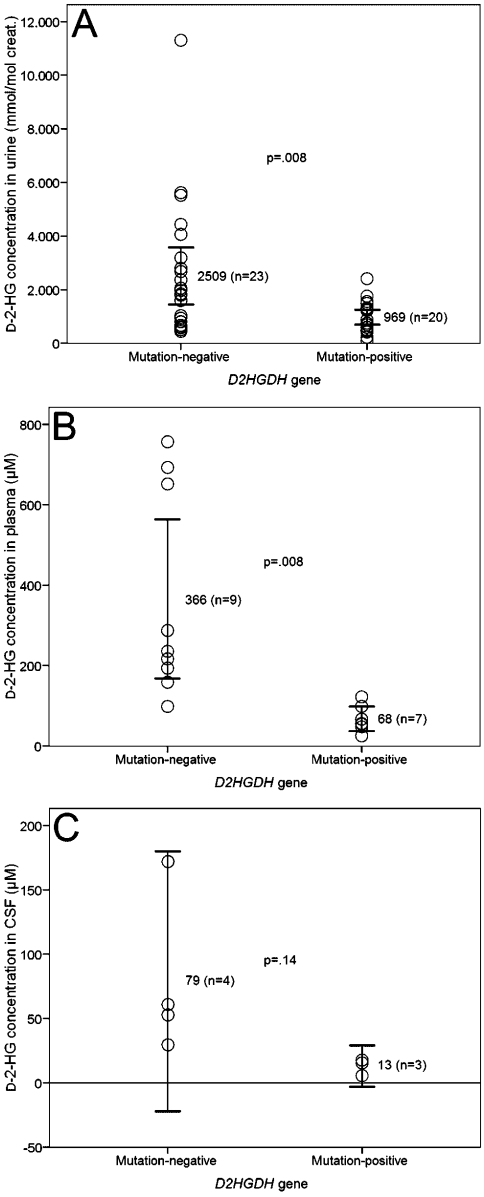

Figure 2. Mean D-2-HG concentrations in (**A**) urine, (**B**) plasma, and (**C**) CSF of D-2-HGA patients. Error bars are 95% confidence intervals and *P*-values are determined by Student *t*-test (two-tailed, assumed unequal variance, $n = \sharp$ of subjects, SPSS 15.0). Normal D-2-HG concentrations: urine < 17.0 mmol/mol creatinine, plasma < 0.9 µM, CSF < 0.3 µM.

located within the mitochondria, it remains possible that idiopathic D-2-HGA is associated with a currently unknown subcellular compartment (i.e., cytosolic) defect involving D-2-HG

Figure 3. Metabolic routes involving D-2-HG: D-2-HG originates from the metabolism of 2-KG via HOT, a byproduct of the oxidation of *gamma*-hydroxybutyrate (GHB) to succinic semialdehyde (SSA). Subsequently, D-2-HG is oxidized to 2-KG via D-2-HGDH, which is deficient in D-2-HGA Type I patients leading to accumulation of D-2-HG in body fluids.

metabolism. Deficiency of the mannose-6-phosphate independent trafficking receptor for β-glucocerebrosidase (LIMP-2) [Reczek et al., 2007] and hyperoxaluria Type I [Danpure, 2004] are examples of metabolic disorders in which enzymes are misdirected to an incorrect cellular location.

Recent isotopomer studies using labeled glucose and glutamic acid have demonstrated that D-2-HG derives from mitochondrial 2-KG in D-2-HGA patients [Struys et al., 2004b]. Hydroxyacid-oxoacid transhydrogenase (HOT), an enzyme described by Kaufman and coworkers, is likely the enzyme responsible for D-2-HG generation (Fig. 3) [Kaufman et al., 1988; Struys et al., 2005c]. Hyperactivity of HOT could conceivably lead to increased levels of D-2-HG, and secondary increases of D-2-HG in succinic semialdehyde dehydrogenase deficiency (gamma-hydroxybutyric aciduria) have been observed [Struys et al., 2006]. Preliminary studies, however, revealed normal HOT activities in homogenates of fibroblasts derived from seven D-2-HGA patients [Struys et al., 2005c], including one D-2-HGA Type I and six idiopathic patients. Mildly increased urinary 2-HG concentration has been observed in 2-ketoglutarate dehydrogenase deficiency, yet no enantiomeric differentiation has been presented [al Aqeel et al., 1994; Kohlschutter et al., 1982]. In these patients, significantly increased 2-KG concentrations likely alter the kinetic equilibrium of the HOT enzyme, leading to increased D-2-HG production (Fig. 3). Altered kinetics may also account for the formation of L-2-HG, linked to nonspecific reduction of 2-KG via L-malate dehydrogenase and subsequent oxidation via L-2-hydroxyglutarate dehydrogenase [Rzem et al., 2007].

In summary, mutations in *D2HGDH* have been identified to be disease causing for about 50% of patients affected with D-2-hydroxyglutaric aciduria. Evidence exists that this form of D-2-HGA is inherited in an autosomal recessive pattern. Whether the *D2HGDH* mutation-negative patients are affected with an autosomal recessive disorder awaits further studies. In the diagnostic workup of D-2-HGA patients, we propose that enzyme studies should be initially performed to select only the D-2-HGDH deficient patients for molecular analysis of the *D2HGDH* gene. We continue to develop novel metabolic and molecular probes to define the underlying genetic abnormality in idiopathic D-2-HGA patients.

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IDH2 mutations in patients with D-2-hydroxyglutaric aciduria

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IDH2 Mutations in Patients with D-2-Hydroxyglutaric Aciduria

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Recent studies in human cancer genetics have led to a resurgence of interest in a group of metabolic enzymes called isocitrate dehydrogenases. A recurrent heterozygous somatic mutation in the gene encoding cytosolic isocitrate dehydrogenase-1 (IDH1) is present in glioblastoma multiforme and alters residue Arg^{132} (R132) in the enzyme's active site (*1*). This mutation disables the enzyme's normal ability to convert isocitrate to 2-ketoglutarate (2-KG) and confers on it a new function: the ability to convert 2-KG to D-2-hydroxyglutarate (D-2-HG) (*2*). Heterozygous mutations that alter residues R140 and R172 of mitochondrial isocitrate dehydrogenase-2 (IDH2), the latter corresponding to R132 of IDH1, have been detected in other tumor types, acute myeloid leukemia and gliomas. These mutations also lead to abnormal production of D-2-HG (*3*, *4*).

This unusual pathophysiological mechanism prompted us to explore whether mutations in *IDH1* or *IDH2* are associated with D-2-hydroxyglutaric aciduria (D-2-HGA) (Mendelian Inheritance in Man no. 600721). D-2-HGA is a rare inherited neurometabolic disorder with a wide clinical spectrum. Although some children with D-2-HGA are asymptomatic, others exhibit characteristics that can include developmental delay, epilepsy, hypotonia, cardiomyopathy, and dysmorphic features. All affected individuals have consistently increased D-2-HG levels in urine, plasma, and cerebrospinal fluid (*5*). About 50% of patients with this disorder, denoted D-2-HGA type I, have autosomal recessive mutations in the gene *D2HGDH* encoding D-2-hydroxyglutarate dehydrogenase (*6*), but the genetic basis of the disease in the remaining patients is unknown (*5*).

We sequenced the open reading frames of *IDH1* and *IDH2* in 17 unrelated idiopathic D-2-HGA patients (i.e., normal D-2-HGDH enzyme activity or no mutations in *D2HGDH* and consistently increased D-2-HG levels in body fluids). No mutations were detected in *IDH1*. In 15 patients, germline mutations were detected in *IDH2*: the known heterozygous G-to-A substitution at position 419 (c.419G>A), resulting in the replacement of Arg^{140} with Gln^{140} (p.R140Q) (*4*), and a novel heterozygous C-to-G substitution at position 418 (c.418C>G), Arg^{140}→Gly^{140} (p.R140G) (Fig. 1 and table S1). Although the D-2-HGDH enzyme functions normally in these patients, the active protein appears to lack the catalytic capacity to oxidize all D-2-HG formed by IDH2 containing the R140 mutation; we thus denote the disorder in these patients as D-2-HGA type II. The higher urinary excretion of D-2-HG in the type II patients compared with that of type I patients (Fig. 1) is best explained by hyperproduction of this metabolite. The involvement of mitochondrial IDH2 is also consistent with the finding that D-2-HG is derived from mitochondrial 2-KG (*7*).

In eight of nine sets of parents, the mutation could not be detected, indicating that the heterozygous mutation arose de novo and that D-2-HGA type II is an autosomal dominant trait. In one family, however, three subsequent affected pregnancies were diagnosed by increased D-2-HG levels in amniotic fluid, suggesting germline mosaicism in the mother who herself had normal urinary D-2-HG levels and showed somatic mosaicism in her blood (Fig. 1).

	D-2-HGA type I	D-2-HGA type II
Number of participants	24	15
Urinary D-2-HG (mmol mol creat.$^{-1}$) [normal amount in controls < 17.0]	Mean = 969 (n = 20)	Mean = 2153 (n = 14)
Gene and accession no.	*D2HGDH* NM_152783.3	*IDH2* NM_002168.2
Type of mutations	Heterogeneous (*5*, *6*)	c.419G>A, R140Q (n = 14) c.418C>G, R140G (n = 1)
Trait	Autosomal recessive	Autosomal dominant
Probability of affected fetus	25%	>0%–50%

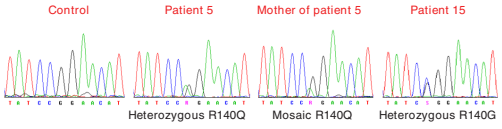

Fig. 1. Characteristics of patients with D-2-HGA types I and II. The living D-2-HGA type II patients (n = 6) range in age from 3 to 22 years. The age of death of the remaining patients (n = 9) ranged from a few months up to 14 years. To date, none of the patients has been diagnosed with cancer. Shown below the table are sequence chromatograms from patients 5 and 15 (table S1), who have heterozygous *IDH2* mutations. Somatic mosaicism for the R140Q mutation was detected in DNA of the mother of patient 5, unlike the other sets of parents in whom the mutation was not detected.

The pathophysiological consequences of increased D-2-HG in both cancer and D-2-HGA remain to be determined. Dang *et al.* have hypothesized that D-2-HG is an "onco-metabolite" that contributes to the formation of gliomas (*2*). However, patients with malignant gliomas and anaplastic astrocytomas that harbor *IDH1* or *IDH2* mutations show improved survival in comparison to patients whose tumors lack these mutations (*1*, *4*). The absence of cancer diagnoses in our D-2-HGA patient population (>85 patients) is also not consistent with the proposed role of D-2-HG as an onco-metabolite, although the 15 D-2-HGA type II patients are young, which may preclude firm conclusions about cancer susceptibility (Fig. 1 and table S1).

An increased incidence of brain tumors has been noted among patients with L-2-hydroxyglutaric aciduria (L-2-HGA) (*8*). L-2-hydroxyglutarate (L-2-HG) is the stereoisomer of D-2-HG, but L-2-HGA is a distinct neurometabolic disease. In contrast to D-2-HGA, L-2-HGA is a leukodystrophy. L-2-HGA manifests in early childhood with slowly progressive neurological symptoms, including psychomotor retardation, cerebellar ataxia, variable macrocephaly, and epilepsy (*9*). The biochemical defect in L-2-HGA is caused by mutations in the *L2HGDH* gene, which encodes an enzyme that specifically degrades the L enantiomer of 2-HG (*9*).

Now that disease-associated mechanisms have been described for nearly all D-2-HGA patients, genetic counseling is expected to be enhanced. Our findings provide additional impetus for investigating the role of D-2-HG in the pathophysiology of inborn errors of metabolism and neoplastic disorders.

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Supporting Online Material

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Supporting Online Material for

IDH2 Mutations in Patients with D-2-Hydroxyglutaric Aciduria

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This PDF file includes:
Supplementary Methods and Materials
Table S1, S2
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Supplementary Methods and Materials

Enrollment and diagnosis of D-2-HGA patients

Enantiomeric separation and quantification of urinary D-2-hydroxyglutarate (D-2-HG) was performed by LC-MS/MS as previously described (**Table S1**)(*S1*). This specialized analysis was preceded by the finding of increased total 2-HG in the urinary organic acid profiling of patients worldwide. Only idiopathic D-2-HGA patients (n=17) in whom both the D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) activity was proven to be normal in cultured cells (i.e. fibroblasts or lymphoblasts (*S2*)), as well as in whom no mutations were detected in the *D-2-hydroxyglutarate dehydrogenase* (*D2HGDH:* NM_152783.3) gene were enrolled in this study (*S3*). Patients were from Australia (n=1), Canada (n=2), UK (n=1) France (n=1), Germany (n=1), Israel (n=2), Netherlands (n=5), Sweden (n=1), USA (n=2), Poland (n=1). Informed consent was waived because of the retrospective nature of the study. The study received Institutional Review Board approval from the VU University Medical Center Amsterdam, Netherlands (registered with the US Office of Human Research Protections under number IORG0002436).

DNA sequence analysis of the open reading frames (ORF) of *IDH1* and *IDH2*.

For the index patients (n=17) all exons and the adjacent splice sites of the ORFs of the *IDH1* (NM_005896.2) and *IDH2* (NM_002168.2) genes were amplified by PCR using the primers described below and analyzed by direct DNA sequence analysis using an M13 Forward and M13 Reverse primer (**Table S2**). The DNA samples of their parents (9 sets of parents) were only investigated for the amplicon comprising R140 (i.e. exon 4). The presence/absence of the mutations was confirmed in independent PCR products. The amplicons were analyzed by capillary electrophoresis using an ABI 3130xl genetic analyzer (Applied Biosystems, Nieuwerkerk a/d lJssel, NL) and assessed using Mutation Surveyor® (Softgenetics, PA, USA). In total two different mutations were detected in 15 out of 17 patients of this series: the known heterozygous c.419G>A, p.R140Q and a novel heterozygous c.418C>G, p.R140G. In addition in one case maternal somatic mosaicsm was detected: the wildtype G allele only drops 30% (drop value 0.3), in heterozygous state this is 50% (drop value 0.5).

Table S1: Characteristics of individual D-2-HGA Type II patients

Subject	Gender	Current age *(yr)*	D-2-HG levels			Mutation IDH2	Cancer diagnosed
			Urine *(mmol mol creat.$^{-1}$)*	Plasma *(µmol L^{-1})*	CSF *(µmol L^{-1})*		
Controls			*<17.0*	*<0.93*	*<0.34*		
1	F	0.4†	5622	-	-	R140Q	No
2	F	22	3193	-	-	R140Q	No
3	F	16	1259 498 1059	-	-	R140Q	No
4	M	0.7†	448	-	-	R140Q	No
5	F	8†	-	-	-	R140Q	No
6	F	7†	1975	757	-	R140Q	No
7	F	4†	585	159	-	R140Q	No
8	F	3	2798	-	-	R140Q	No
9	M	14†	4067	217	61	R140Q	No
10	F	<1†	1127 7751	662 640 654	172	R140Q	No
11	M	0.4†	619	-	-	R140Q	No
12	M	3	514	99		R140Q	No
13	M	>8†	2220 3142	147 241	67 39	R140Q	No
14	F	11	1732 1463	244 228 150	-	R140Q	No
15	M	19	676 666	-	-	R140G*	No

† = deceased at the indicated age
* = novel mutation

Table S2: List of primers used to amplify the coding exons of *IDH1* and *IDH2*

Primer	Sequence 5' - 3'	Product size
IDH1_EX3F	GTAAAACGACGGCCAGACCGCGTGTGAAACATAACA	520
IDH1_EX3R	CAGGAAACAGCTATGATGAATTCCTGGTCTTGAGGGA	
IDH1_EX4F	GTAAAACGACGGCCAGTGGTGTACTCAGAGCCTTCG	560
IDH1_EX4R	CAGGAAACAGCTATGACATACCTTGCTTAATGGGTGT	
IDH1_EX5F	GTAAAACGACGGCCAGTGCCCTATTTGTCTAGGTGTC	388
IDH1_EX5R	CAGGAAACAGCTATGAGTCAAGTTTCGGGTTTTGCA	
IDH1_EX6F	GTAAAACGACGGCCAGGTGGTGGGTGATTTTAGCCT	386
IDH1_EX6R	CAGGAAACAGCTATGACCCAGAATCATAGGGATAGGG	
IDH1_EX7F	GTAAAACGACGGCCAGCTGTTTGGGACAAGCAGATG	341
IDH1_EX7R	CAGGAAACAGCTATGACTACAAAACTCCCCTTCCCA	
IDH1_EX8F	GTAAAACGACGGCCAGTGGTGATTCCATGTGCTCTT	396
IDH1_EX8R	CAGGAAACAGCTATGAACACAAAACACTGAGCAGCC	
IDH1_EX9F	GTAAAACGACGGCCAGGGGGAACTATGAGACATTTGG	354
IDH1_EX9R	CAGGAAACAGCTATGAATAATTCCAGAAAGCACCGA	
IDH1_EX10F	GTAAAACGACGGCCAGCATGAATGCGTTTTCTTCCA	341
IDH1_EX10R	CAGGAAACAGCTATGATTTTGCCTTTATCCTTGAGTG	
IDH2_Ex_1F	GTAAAACGACGGCCAGAGGCCCAGCGTTAGCCCG	290
IDH2_Ex_1R	CAGGAAACAGCTATGACAGCCTGGGAAGCCGCCA	
IDH2_Ex2_F	GTAAAACGACGGCCAGTGTCTCCTGGTGAAGGGC	294
IDH2_Ex2_R	CAGGAAACAGCTATGAGTCCAGAAGACCCTGTGGG	
IDH2_Ex3_F	GTAAAACGACGGCCAGTTCTTATCCTGCCCTTTGTTG	368
IDH2_Ex3_R	CAGGAAACAGCTATGAATGAGTGACATGGCCAACTG	
IDH2_Ex4_F	GTAAAACGACGGCCAGTTGTTGCTTGGGGTTCAAAT	569
IDH2_Ex5_R	CAGGAAACAGCTATGAAAGGAAAGCCACGAGACAGA	
IDH2_Ex6_F	GTAAAACGACGGCCAGGGGGTACCGTTCCTGGAG	710
IDH2_Ex7_R	CAGGAAACAGCTATGAGCAATGAGTCCTGCTCCACT	
IDH2_Ex8_F	GTAAAACGACGGCCAGGTTGTAGAGGGCAGCAGGG	583
IDH2_Ex9_R	CAGGAAACAGCTATGAGGATGGGGCAGAATGAGAC	
IDH2_Ex10_F	GTAAAACGACGGCCAGGTGAGCATGGAGGGAGAGG	291
IDH2_Ex10_R	CAGGAAACAGCTATGAGGGGACTTTAGGAGGGGTC	
IDH2_Ex11_F	GTAAAACGACGGCCAGGTTCTGATGCCCAAGCTCAG	328
IDH2_Ex11_R	CAGGAAACAGCTATGATAGAAAGGCCTCCAGAGAGG	
Forward M13F	GTAAAACGACGGCCAG	
Reverse M13R	CAGGAAACAGCTATGA	

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A lymphoblast model for IDH2 gain-of-function activity in D-2-hydroxyglutaric aciduria type II: Novel avenues for biochemical and therapeutic studies

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A lymphoblast model for IDH2 gain-of-function activity in D-2-hydroxyglutaric aciduria type II: Novel avenues for biochemical and therapeutic studies

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ABSTRACT

The recent discovery of heterozygous isocitrate dehydrogenase 2 (IDH2) mutations of residue Arg^{140} to Gln^{140} or Gly^{140} (IDH2$^{wt/R140Q}$, IDH2$^{wt/R140G}$) in D-2-hydroxyglutaric aciduria (D-2-HGA) has defined the primary genetic lesion in 50% of D-2-HGA patients, denoted type II. Overexpression studies with IDH1^{R132H} and IDH2^{R172K} mutations demonstrated that the enzymes acquired a new function, converting 2-ketoglutarate (2-KG) to D-2-hydroxyglutarate (D-2-HG), in lieu of the normal IDH reaction which reversibly converts isocitrate to 2-KG. To confirm the IDH2$^{wt/R140Q}$ gain-of-function in D-2-HGA type II, and to evaluate potential therapeutic strategies, we developed a specific and sensitive IDH2$^{wt/R140Q}$ enzyme assay in lymphoblasts. This assay determines gain-of-function activity which converts 2-KG to D-2-HG in homogenates of D-2-HGA type II lymphoblasts, and uses stable-isotope-labeled 2-keto[3,3,4,4-$^2H_4$]glutarate. The specificity and sensitivity of the assay are enhanced with chiral separation and detection of stable-isotope-labeled D-2-HG by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Eleven potential inhibitors of IDH2$^{wt/R140Q}$ enzyme activity were evaluated with this procedure. The mean reaction rate in D-2-HGA type II lymphoblasts was 8-fold higher than that of controls and D-2-HGA type I cells (14.4 nmol h^{-1} mg protein^{-1} vs. 1.9), with a corresponding 140-fold increase in intracellular D-2-HG level. Optimal inhibition of IDH2$^{wt/R140Q}$ activity was obtained with oxaloacetate, which competitively inhibited IDH2$^{wt/R140Q}$ activity. Lymphoblast IDH2$^{wt/R140Q}$ showed long-term cell culture stability without loss of the heterozygous IDH2$^{wt/R140Q}$ mutation, underscoring the utility of the lymphoblast model for future biochemical and therapeutic studies.

1. Introduction

The discovery of recurrent mutations in isocitrate dehydrogenase 1 (IDH1) in patients with glioblastoma multiforme has rekindled interest in NADP(H)-dependent isocitrate dehydrogenase enzymes [1]. A high incidence of specific mutations in the active sites of cytosolic and peroxisomal IDH1 (OMIM ID: 147700) (residue Arg^{132}/R132) and the mitochondrial homologue IDH2 (OMIM ID: 147650) (residues Arg^{140}/R140 and Arg^{172}/R172) are found in a wide variety of hematologic malignancies and solid tumors [2]. These mutations alter the enzymes normal capacity to convert isocitrate to 2-ketoglutarate (2-KG) and confer on it a new function which converts 2-KG to D-2-hydroxyglutarate (D-2-HG), leading to accumulation of D-2-HG in solid tumor cells and in plasma of patients with acute myeloid leukemia [3,4].

Supraphysiological D-2-HG concentrations in body fluids are the biochemical hallmark of the inborn errors of metabolism D-2-hydroxyglutaric aciduria (D-2-HGA) type I (OMIM ID: 600721) and type II (OMIM ID: 613657). D-2-HGA type I is caused by various homozygous/compound heterozygous mutations/deletions in the *D2HGDH* gene encoding for D-2-hydroxyglutarate dehydrogenase (D-2-HGDH, OMIM ID: 609186) [5], whereas D-2-HGA type II is caused primarily by the heterozygous *de novo* IDH2$^{wt/R140Q}$ mutation [6]. Thus far only one patient was detected with an IDH2$^{wt/R140G}$ mutation. The phenotype of D-2-HGA has a broad clinical spectrum ranging from asymptomatic to a severe clinical presentation with heterogeneous MRI abnormalities observed [7–10]. Another form of D-2-HGA is associated with spondyloenchondromatosis, but the etiology is unknown [11]. The chiral

Abbreviations: 2-KG, 2-ketoglutarate; $^2H_4$-2-KG, 2-keto[3,3,4,4-$^2H_4$]glutarate; $^2H_4$-D-2-HG, D-2-[3,3,4,4-$^2H_4$]hydroxyglutarate; D-2-HG, D-2-hydroxyglutarate; D-2-HGA, D-2-hydroxyglutaric aciduria; D-2-HGDH, D-2-hydroxyglutarate dehydrogenase; Datan, (+)-O,O′-Diacetyl-L-tartaric anhydride; HOT, hydroxyacid-oxoacid transhydrogenase; IDH, isocitrate dehydrogenase; IDH1$^{wt/wt}$, isocitrate dehydrogenase 1 wild type; IDH2$^{wt/R140Q}$, heterozygous isocitrate dehydrogenase 2 mutation of residue Arg^{140} to Gln^{140}; L-2-HG, L-2-hydroxyglutarate; L-2-HGA, L-2-hydroxyglutaric aciduria; L-2-HGDH, L-2-hydroxyglutarate dehydrogenase; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry

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counterpart of D-2-HG, L-2-hydroxyglutarate (L-2-HG), is increased in patients with L-2-hydroxyglutaric aciduria (L-2-HGA, OMIM ID: 236792), a leukoencephalopathy associated with mutations in the *L2HGDH* gene encoding for L-2-hydroxyglutarate dehydrogenase (L-2-HGDH, OMIM ID: 609584) [12–14]. Whereas an increased incidence of brain tumors is reported in L-2-HGA [15], currently no neoplastic malignancies have been reported in D-2-HGA.

A specific enzyme assay using enantiomerically pure stable-isotope-labeled D-2-HG was developed to assess the enzyme activity of D-2-HGDH in cultured fibroblasts and lymphoblasts, verifying that D-2-HGDH was impaired in D-2-HGA type I patients [16]. An enzyme assay to assess IDH2$^{wt/R140Q}$-mutant enzyme activity in cultured human cells from D-2-HGA type II patients has not been described. Several groups used spectrophotometric assays to study IDH1^{R132H}, IDH1^{R132C}, IDH2^{R140Q}, IDH2^{R140W}, and IDH2^{R172K} enzyme activities via the NADP (H) couple in overexpressed cells [3,4,17], yet this approach suffered from high background issues in extracts of cultured human cells. Pietrak et al. improved the assay's characteristics by employing tandem mass spectrometry to quantify 2-hydroxyglutarate, however without separation of D- and L-2-HG analytes [18]. We sought to develop a more robust assay system which could both verify the presence of the IDH2$^{wt/R140Q}$ gain-of-function activity and simultaneously investigate potential therapeutic inhibitors which might quantitatively decrease D-2-HG production. Accordingly, we developed a new assay which determines IDH2$^{wt/R140Q}$ activity in lymphoblast extracts using stable-isotope-labeled 2-keto[3,3,4,4-$^2H_4$]glutarate ($^2H_4$-2-KG) as substrate. The reaction product, D-2-[3,3,4,4-$^2H_4$]hydroxyglutarate ($^2H_4$-D-2-HG), is specifically detected by derivatization and chiral separation of D-2-HG and L-2-HG using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Further, this new assay facilitated the preclinical evaluation of several endogenous metabolites for their ability to inhibit IDH2$^{wt/R140Q}$ activity.

2. Materials and methods

2.1. Lymphoblast cell lines and protein extraction

All lymphoblast cell lines were obtained from Epstein–Barr virus transfected lymphocytes and were anonymized. Five cell lines were obtained from D-2-HGA type II patients which all carried the heterozygous *IDH2* c.[419 G>A], p.[Arg140Gln] (IDH2$^{wt/R140Q}$) mutation and cell culture stability was evaluated by confirmation of the presence of the heterozygous IDH2$^{wt/R140Q}$ mutation by DNA sequencing analysis. Additionally, five cell lines obtained from healthy individuals served as controls, supplemented with two cell lines obtained from D-2-HGA type I patients which carried compound heterozygous mutations in *D2HGDH* associated with impaired D-2-HGDH enzyme activity [5].

Lymphoblasts were grown in RPMI 1640 culture medium supplemented with 1% penicillin/streptomycin and 8% fetal bovine serum (all from Invitrogen) until a sufficient cell suspension was achieved to obtain a cell pellet containing ~10^6 cells. Subsequently, cells were harvested by centrifugation (6 min 340 g), washed twice with Hank's balanced salt solution and stored as a dry cell pellet at −80 °C prior to analysis.

Cell pellets were suspended in 250 μL M-PER containing 1% HALT protease/phosphatase inhibitor (Thermo Scientific), sonicated using a Bandalin Sonopuls mini 20 titanium 1.5 mm probe for 10 s at 90% power while cooled on ice, followed by centrifugation of the cell extract for 15 min at 14,000 g. The clarified supernatant was transferred to a 1.5 mL Eppendorf tube and gently mixed. Protein concentration was determined with a Pierce BCA protein assay (Sigma-Aldrich).

2.2. Incubation and UPLC-MS/MS analysis

The assay was performed in duplicate for 30 min at 37 °C in 150 μL buffer pH = 7.5 containing 100 mM Tris–HCl, 5 mM $MnCl_2$, 0.2 mM NADPH (Sigma-Aldrich), 15 mM 2-keto[3,3,4,4-$^2H_4$]glutarate ($^2H_4$-2-KG) (Euriso-Top, Gif sur Yvette, France) with a final protein concentration of 0.40 mg mL^{-1}. The reaction was terminated by cooling the samples on ice directly followed by protein removal via centrifugation with Microcon Ultracel YM-10 kDa filters (Millipore) in a pre-chilled centrifuge (15 min 20,000 g 4 °C). Twenty microliters of filtrate was transferred to a 1.5 mL HPLC vial, mixed with 200 pmol D/L-2-hydroxy[2-$^2H_1$]glutarate as internal standard in 200 μL methanol, and dried with $N_{2(g)}$ at 40 °C ($^2H_1$-D/L-2-HG, made in-house from unlabeled 2-KG by chemical reduction with zinc in deuterated water [24]). Subsequently, 50 μL (+)-O,O′-Diacetyl-L-tartaric anhydride (Datan, Aldrich) derivative reagent (50 mg mL^{-1} dissolved in dichloromethane:acetic acid 80:20 (v/v)) was added, and the vials were capped and heated 30 min 75 °C. Finally, the samples were dried with $N_{2(g)}$ at 40 °C and dissolved in 500 μL mobile phase A (details below). Calibration lines of 10 to 400 pmol for each metabolite were composed of unlabeled D/L-2-HG (Sigma) and $^2H_4$-D/L-2-HG (made in-house from $^2H_4$-2-KG [24]). The substrate $^2H_4$-2-KG is converted by IDH2$^{wt/R140Q}$ into $^2H_4$-D-2-HG (Fig. 1), and the latter is determined by derivatization with Datan, followed by UPLC-MS/MS analysis [19], as a measure of the enzyme activity. Since the method allows the separation of D- and L-2-hydroxyglutarate, account was taken of the fact that D-2-hydroxyglutarate amounts to half of the total amount of 2-hydroxyglutarate in the standards.

For chromatographic analysis, a Waters Acquity UPLC was equipped with an HSS T3 column (2.1 × 100 mm 1.8 μm particle size) running at 0.4 mL min^{-1} 97% mobile phase A (120 mg mL^{-1} ammonium formate pH = 3.25) and 3% acetonitrile followed by a 40% acetonitrile wash step. Injection volume was 5 μL and detection was performed with an Applied Biosystems 4000 Q Trap mass spectrometer equipped with an ESI Turbo Spray interface running in negative MRM mode: transitions (m/z) D/L-2-HG = 363.0 > 146.9; $^2H_1$-D/L-2-HG = 364.0 > 147.9; $^2H_4$-D/L-2-HG = 367.0 > 150.9. Other settings included: CUR = 10; TEM = 100; GS1 = 40; GS2 = 50; ihe = ON; IS = −4500; CAD = 3; DP = −20; EP = −5; CE = −12.5; CXP = −10. The retention times were 2.8 min for L-2-HG and 3.4 min for D-2-HG (Fig. S1). Raw data processing was performed using Analyst 1.4.2./MS Excel and concentrations were extrapolated from calibration lines (Fig. S2).

2.3. IDH2$^{wt/R140Q}$ inhibition

Eleven (endogenous) metabolites were selected based upon their structural similarities with 2-KG, D-2-HG or isocitrate to test their capacity to reduce $^2H_4$-D-2-HG production by IDH2$^{wt/R140Q}$: D-2-HG, L-2-HG, L-isocitrate (D_s-(+)-*threo*-isocitric acid monopotassium salt), citrate, L-glutamate, L-glutamine, L-malate, D-malate, oxaloacetate, acetoacetate and acetoacetic acid methyl ester. Inhibitors were evaluated at up to ~30 mM concentration. All other parameters were held constant, as was the $^2H_4$-2-KG substrate concentration of 15 mM. The pH of the reaction mixture was monitored before and after incubation to verify constant experimental conditions, especially important since IDH2$^{wt/R140Q}$ is sensitive to small pH changes. The K_i of oxaloacetate was evaluated by determining the K_M of $^2H_4$-2-KG with 0.0, 5.8 and 11.7 mM oxaloacetate supplemented in the reaction mixture.

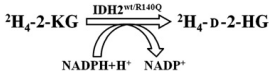

Fig. 1. Reaction mechanism catalyzed by IDH2$^{wt/R140Q}$ converting stable isotope labeled $^2H_4$-2-KG into $^2H_4$-D-2-HG with NADPH as electron donor.

3. Results

3.1. IDH2$^{wt/R140Q}$ gain-of-function enzyme assay

Optimal pH and concentrations of assay components (TRIS, $MnCl_2$, NADPH) were determined under conditions of the highest reaction rates of IDH2$^{wt/R140Q}$ in cell lysates of D-2-HGA type II lymphoblasts (data not shown). The reaction rate was stable over 60 min and the reaction dependence on protein content was linear to 1.14 mg protein mL^{-1} (data not shown). Substrate dependence for IDH2$^{wt/R140Q}$ was determined in D-2-HGA type II cells with $K_{M,\ 2\text{-}KG} = 1.7$ mM and $V_{max} = 13.9$ nmol h^{-1} mg protein^{-1}. The observed $K_{M,\ 2\text{-}KG}$ is of the same magnitude as previously reported for IDH1^{R132H} overexpressed in cells: $K_{M,\ 2\text{-}KG} = 0.965$ mM [3] and $K_{M,\ 2\text{-}KG} = 0.7$ mM [18], whereas no $K_{M,\ 2\text{-}KG}$ has been previously reported for IDH2-mutants. $^2H_4$-D-2-HG production was also detected in control cells as well, produced by an unidentified enzyme, which had different characteristics of substrate dependence as compared to IDH2$^{wt/R140Q}$: $K_{M,\ 2\text{-}KG} = 0.7$ mM and $V_{max} = 2.0$ nmol h^{-1} mg protein^{-1} (Fig. 2).

The mean reaction rate detected in control (n = 5) and D-2-HGA type I (n = 2) lymphoblasts was 1.8 and 1.9 nmol h^{-1} mg protein^{-1} respectively, whereas in D-2-HGA type II (n = 5) the mean reaction rate was ~8 times higher: 14.4 nmol h^{-1} mg protein^{-1} (Fig. 3). This resulted in increased intracellular D-2-HG concentrations in D-2-HGA type II cells when compared to controls/D-2-HGA type I cells. The mean endogenous D-2-HG (nmol mg protein^{-1}) values were: controls = 0.11, D-2-HGA type I = 1.80, and D-2-HGA type II = 15.06 (Fig. 4). The higher concentration of D-2-HG in D-2-HGA type II compared to type I is consistent with the trend observed in body fluids as previously reported [5,6]. Conversely, the intracellular L-2-HG concentrations were comparable among all twelve cell lines, verifying the independence of this metabolite from D-2-HG (Fig. 5). Furthermore, small amounts of $^2H_4$-L-2-HG were detected, which were also equivalent between all cell lines. $^2H_4$-L-2-HG most likely originates from the nonspecific conversion of $^2H_4$-2-KG via L-malate dehydrogenase, an NADH-dependent reaction [20]. In support of this observation, when NADPH was replaced by NADH in the assay buffer with D-2-HGA type II cells, $^2H_4$-L-2-HG production increased 24-fold, whereas $^2H_4$-D-2-HG production decreased 9-fold (data not shown). The IDH2$^{wt/R140Q}$ assay demonstrated little diagnostic sensitivity in fibroblast homogenates in its ability to distinguish between IDH2$^{wt/R140Q}$ enzyme activity and that observed in controls. The $^2H_4$-D-2-HG production in D-2-HGA type II lysates was only twice that observed in controls/D-2-HGA type I cells, which accounted for the observed intracellular D-2-HG accumulation as well (data not shown).

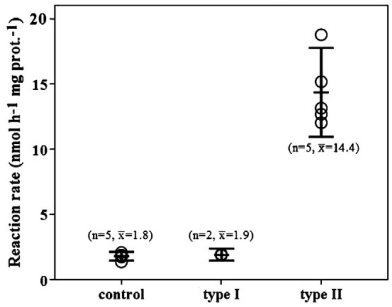

Fig. 3. Reaction rates (nmol h^{-1} mg protein^{-1}) determined in cell homogenates of controls (n = 5, mean = 1.8), D-2-HGA type I (n = 2, mean = 1.9) and D-2-HGA type II cells (n = 5, mean = 14.4), error bars: 95% CI (SPSS 15.0).

3.2. IDH2$^{wt/R140Q}$ inhibition

From eleven metabolites used to screen for inhibition of IDH2$^{wt/R140Q}$, oxaloacetate was the most potent: the $^2H_4$-D-2-HG production was more than 50% reduced when equal concentrations (15 mM) of substrate and inhibitor were used (Fig. 6). Decreasing reaction rates and increasing K_M for $^2H_4$-D-2-HG were observed when 2-KG substrate dependence was tested for IDH2$^{wt/R140Q}$ with varying oxaloacetate concentration and a $K_{i,\ oxaloacetate}$ of 3.9 mM was computed from the data (Fig. 6). Oxaloacetate functions as a competitive inhibitor in IDH2$^{wt/R140Q}$ with a slightly lower selectivity compared to 2-KG, since $K_{i,\ oxaloacetate}/K_{M,\ 2\text{-}KG} = 2$.

4. Discussion

Biochemical mechanisms for heterozygous IDH1 and IDH2 mutations have been recently summarized. IDH1 and IDH2 are NADP(H)-dependent homodimers catalyzing the reversible conversion of isocitrate to 2-KG. Zhao et al. observed that heterodimer formation between a wild type and mutant subunit (IDH1$^{wt/R132H}$) resulted in considerable loss of isocitrate to 2-KG catalytic activity, while

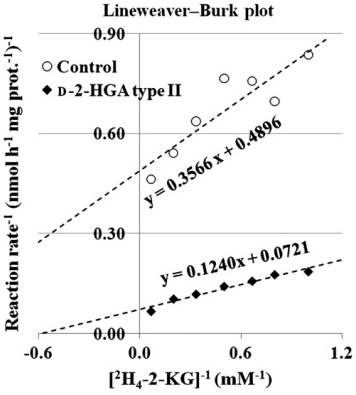

Fig. 2. Lineweaver-Burk plot showing the reciprocal reaction rate as a function of reciprocal $^2H_4$-2-KG concentration for: (♦) IDH2$^{wt/R140Q}$ activity in D-2-HGA type II cell homogenate [$K_M = 1.7$ mM and $V_{max} = 13.9$ nmol h^{-1} mg protein^{-1}] and (○) unidentified enzyme in control cell homogenate [$K_M = 0.7$ mM and $V_{max} = 2.0$ nmol h^{-1} mg protein^{-1}].

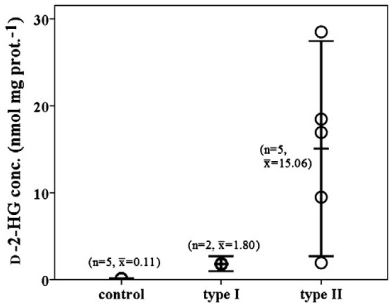

Fig. 4. Endogenous intracellular D-2-HG concentrations (nmol mg protein^{-1}) determined in cell homogenates of controls (n = 5, mean = 0.11), D-2-HGA type I (n = 2, mean = 1.80) and D-2-HGA type II (n = 5, mean = 15.06), error bars: 95% CI (SPSS 15.0).

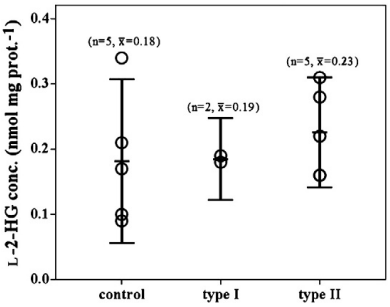

Fig. 5. Endogenous intracellular L-2-HG concentrations (nmol mg protein^{-1}) determined in cell homogenates of controls (n = 5, mean = 0.18), D-2-HGA type I (n = 2, mean = 0.19) and D-2-HGA type II (n = 5, mean = 0.23), error bars: 95% CI (SPSS 15.0).

the mutant IDH1$^{R132H/R132H}$ homodimer is completely devoid of this activity [21]. D-2-HG is formed from 2-KG and NADPH [3,4] in cells that overexpress IDH1^{R132H} and IDH2^{R172K}. Pietrak et al. demonstrated that IDH1$^{wt/R132H}$ subunits function independently, resulting in a coupled reaction which produces 2-HG from isocitrate and $NADP^+$ [18], as had been previously hypothesized by Dang and Ward et al. Unfortunately, chiral differentiation of product 2-HG was not performed to differentiate the level of D- and L-2-HG production. In the coupled reaction sequence, isocitrate and $NADP^+$ are converted into 2-KG and NADPH via IDH1^{wt}-subunit catalysis, with subsequent production of 2-HG and $NADP^+$ via IDH1^{R132H}-subunit catalysis. Further, Pietrak and co-workers demonstrated production of 2-HG from 2-KG via IDH1 wild type (IDH1$^{wt/wt}$) catalysis, a reaction strongly inhibited by isocitrate. This reaction might represent an additional source of D-2-HG in humans in conjunction with the reaction catalyzed by hydroxyacid-oxoacid transhydrogenase (HOT, OMIM ID: 611083) [22]. HOT primarily converts gamma-hydroxybutyrate (GHB) to succinic semialdehyde coupled to the reduction of 2-KG to D-2-HG. However, HOT has specificity towards several other GHB-like metabolites which serve as electron donors as well. These reactions catalyzed by IDH$^{wt/wt}$ and HOT likely contribute to endogenous D-2-HG production observed in the IDH2$^{wt/R140Q}$ enzyme assay (Fig. 2), and most likely contributed a high proportion of the background activity in assays employing fibroblast extracts.

The concentration of D-2-HG is increased 140 fold in lymphoblasts from patients with IDH2$^{wt/R140Q}$-mutations compared to controls, whereas the enzymatic activity of D-2-HG production in cell free systems is increased by only 8-fold. Perhaps, the larger difference observed for intact cells is due to the fact that D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) consumes essentially all the D-2-HG that is formed in control cells, but due to limited capacity [23], it is incapable of metabolizing all of the D-2-HG formed by IDH2$^{wt/R140Q}$. Accordingly, the amount of D-2-HG that accumulates in cells that are deficient in D-2-HGDH (and have a non-mutated IDH2) amounts to about 1/8th of that found in cells from patients with mutated IDH2. Additionally, our in vitro enzyme assay conditions may not exactly recapitulate those in the mitochondrial compartment, and thus the IDH2$^{wt/R140Q}$ reaction rate determined in vitro may be an underestimation of the endogenous intracellular rate.

The IDH2$^{wt/R140Q}$ assay was useful for exploring preclinical therapeutic interventions as well. Oxaloacetate acted as a competitive inhibitor for the conversion of 2-KG to D-2-HG. The comparable affinity of IDH2$^{wt/R140Q}$ for oxaloacetate and 2-KG likely relates to their structural similarity. The assay is also potentially useful to evaluate the capacity of mutated IDH1 or IDH2 to produce D-2-HG in extracts of cancerous cells and thereby take into account the amount of 'active' mutated IDH, which may vary among patients. Additionally, the assay can possible be employed to detect the effect of other, as yet undetected mutations, which might also convert IDH to a D-2-HG-producing enzyme.

In conclusion, the IDH2$^{wt/R140Q}$ gain-of-function enzyme assay we have developed is readily applicable to cultured lymphoblasts derived from D-2-HGA type II patients. Chiral separation and quantification of stable-isotope-labeled D-2-hydroxyglutarate by UPLC-MS/MS provided a highly specific and sensitive method, and an assay which facilitates rapid identification of D-2-HGA type II cells with IDH2$^{wt/R140Q}$ gain-of-function. Perhaps most important, heterozygous IDH2$^{wt/R140Q}$ mutations and gain-of-function activity are preserved intracellularly following long-term storage and cell culture, highlighting the utility of the lymphoblast model for future biochemical and therapeutic explorations.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2011.08.006.

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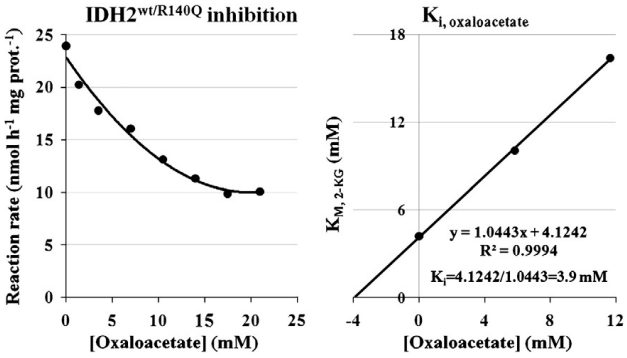

Fig. 6. [left] Inhibition of IDH2$^{wt/R140Q}$ reaction rate as a function of oxaloacetate concentration assessed in D-2-HGA type II cell homogenates using standard assay conditions ($^2H_4$-2-KG substrate conc. = 15 mM); [right] Observed $K_{M,\ 2\text{-}KG}$ with different oxaloacetate concentrations (0, 5.8 and 11.7 mM). The x-axis intercept of the regression line represents -K_i: $K_{i,\ oxaloacetate}$ = 3.9 mM.

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Progress in understanding 2-hydroxyglutaric acidurias

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6

Introduction

The enantiomers D-2- and L-2-hydroxyglutaric acid (D-2-HG and L-2-HG) were recognized as normal constituents of human urine in 1977 (Gregersen et al. 1977). Three years later two novel inborn errors of metabolism were identified and published simultaneously in *Journal of Inherited Metabolic Disease*. Chalmers and co-workers identified the first D-2-hydroxyglutaric aciduria (D-2-HGA) patient (Chalmers et al. 1980), whereas Duran et al. described the first patient affected with L-2-hydroxyglutaric aciduria (L-2-HGA) (Duran et al. 1980), appointing the metabolic hallmarks to highly elevated D-2-HG and L-2-HG in these disorders. In 2000, Muntau and co-workers recognized a third biochemical variant of 2-hydroxyglutaric aciduria (2-HGA) by describing three patients with increased levels of both D-2-HG and L-2-HG, which was denoted "combined D,L-2-hydroxyglutaric aciduria" (D,L-2-HGA) (Muntau et al. 2000). Major breakthroughs were achieved in 2004 by identifying two genes: *D2HGDH* encoding D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) (Achouri et al. 2004) and *L2HGDH* encoding L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) (Rzem et al. 2004; Topcu et al. 2004). In many D-2-HGA and nearly all L-2-HGA patients mutations were detected in these genes unraveling the etiology (Struys et al. 2005b; Steenweg et al. 2010). However, in 50% of the D-2-HGA patient population no mutations were detected in *D2HGDH* (Kranendijk et al. 2010a) and the genetic defect was not elucidated until 2010. We then detected the gain-of-function mutation in *isocitrate dehydrogenase 2* (*IDH2*) gene which was causative for the D-2-HG accumulation in these patients (Kranendijk et al. 2010b).

In the light of the above described events, this *Review* covers current metabolic, enzymatic, genetic and clinical proceedings of the *organic acidurias* D-2-HGA, L-2-HGA and D,L-2-HGA. Perspectives of future research and therapeutic explorations are briefly discussed as well.

Enantiomeric D,L-2-hydroxyglutaric acid and its origin

The chemical structure of 2-hydroxyglutaric acid (2-HG) is a five-carbon dicarboxylic acid with a hydroxyl group positioned at the second carbon (Figure 1). Due to the chiral center present at the second carbon, two different three-dimensional (3D) structures exist: D-2-HG and L-2-HG are "non-superimposable" mirror images (Figure 2). The systemic name for D-2-HG is (R)-2-hydroxypentanedioic acid and for L-2-HG is (S)-2-hydroxypentanedioic acid. Enantiomers share many identical chemical and physical properties including melting point, mass, solubility and pKa, but due to their different 3D-structure enzymes interact highly specific to one or the other.

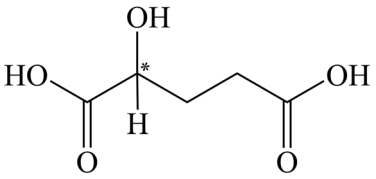

Figure 1: 2-hydroxyglutaric acid with a chiral center at the 2nd carbon (*).

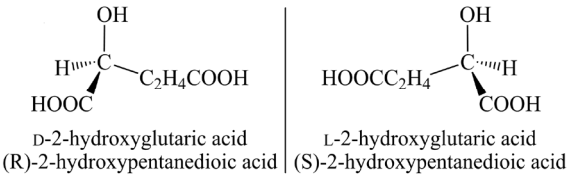

Figure 2: Enantiomers D- and L-2-hydroxyglutatic acid (D-2-HG and L-2-HG, systemic IUPAC names included).

Experiments with human lymphoblasts obtained from D-2-HGA patients incubated with [$^{13}C_6$]glucose or [$^{2}H_5$]glutamic acid revealed that within mitochondria 2-ketoglutarate (2-KG), a tricarboxylic acid (TCA) cycle metabolite, is converted to D-2-HG (Struys et al. 2004b). Subsequent studies demonstrated the presence of hydroxyacid-oxoacid transhydrogenase (HOT) activity in human cells identifying the first enzyme able to produce D-2-HG in humans (Struys et al. 2005c). HOT serves a coupled reaction converting γ-hydroxybutyrate (GHB) to succinic semialdehyde (SSA) parallel with the 2-KG to D-2-HG conversion (Figure 3).

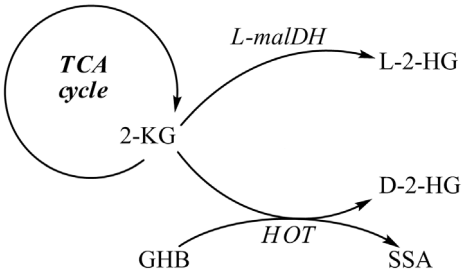

Figure 3: Enzymes L-malDH and HOT are responsible for production of D-2-HG and L-2-HG production from 2-KG.

Incubation experiments using lymphoblasts derived from L-2-HGA patients with [$^{13}C_6$]glucose and [$^{2}H_5$]glutamic acid determined that mitochondrial 2-KG is also the precursor for L-2-HG (Struys et al. 2007). L-malate dehydrogenase (L-malDH) is highly specific towards the reversible conversion of L-malate into oxaloacetate, but Rzem and coworkers determined also activity of L-malDH towards 2-KG, which is currently the only known reaction accountable for L-2-HG production in humans (Rzem et al. 2007) (Figure 3).

2-Hydroxyglutaric aciduria diagnosis

The initiation of diagnosing patients with 2-hydroxyglutaric aciduria starts with the physician who encounters a patient with an inexplicable developmental delay and/or other neurological dysfunction of unknown etiology and suspects a metabolic disorder. Initial diagnosis is in some cases supported with abnormal MRI findings. Routine urinary organic acid screening with gas chromatography-mass spectrometry performed in metabolic centers may than reveal increased 2-HG, but the specific chiral identity is still unknown. Although, the clinical picture often points specifically to either D-2-HGA or L-2-HGA, chiral differentiation performed with gas chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry (LC-MS/MS) concludes the diagnosis with the detection of increased D-2-HG or L-2-HG (Gibson et al. 1993b; Struys et al. 2004a). Additionally, amino acid screening of plasma and cerebrospinal fluid (CSF) may reveal mildly increased lysine levels in L-2-HGA. Subsequently, enzymatic and genetic studies can be performed to confirm the diagnosis of L-2-HGA and differentiate between D-2-HGA type I or D-2-HGA type II, which is important for genetic counseling and prenatal diagnosis in subsequent pregnancies (Steenweg et al. 2010; Kranendijk et al. 2010a; Kranendijk et al. 2010b). Whereas increased L-2-HG levels are only seen in the disorder L-2-HGA and D,L-2-HGA, increased D-2-HG is not only found in D-2-HGA type I and type II but several other disorders may show (secondary) elevated D-2-HG as well, which will be discussed in a separate section of this *Review*. It has been reported that potential false positive diagnosis of D-2- or L-2-HGA may result from poor preservation of (urine) samples due to nonenzymatic conversion of 2-KG to D/L-2-HG or bacterial/fungal growth in urine excreting D/L-2-HG (Kumps et al. 2002), however we never experienced such an observation our laboratory.

D-2-hydroxyglutaric aciduria type I and type II

Recent discoveries in D-2-hydroxyglutaric aciduria (D-2-HGA) revealed the presence of two approximately equal sized groups, type I and type II, encompassing >95% of the patient population. D-2-HGA type I is caused by mutations in the gene *D2HGDH* encoding the enzyme D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) which cause impaired enzyme activity (Kranendijk et al. 2010a). D-2-HGA type II originates from a specific gain-of-function mutation in *IDH2* causing overproduction of D-2-hydroxyglutaric acid (D-2-HG) (Kranendijk et al. 2010b). Both mechanisms result in supraphysiological D-2-HG concentrations in urine, plasma and CSF, which is the biochemical hallmark of these diseases.

Clinical manifestations in D-2-HGA

Most recent extensive clinical descriptions of D-2-HGA date back to 1999, in which D-2-HGA was recognized to be a distinct neurometabolic disorder with a mild and a severe phenotype. Psychomotor retardation, epilepsy and hypotonia were the most frequent findings (van der Knaap et al. 1999a; van der Knaap et al. 1999b). With the description of D-2-HGA type I (*D2HGDH* mutations, MIM# 600721) and D-2-HGA type II (gain-of-function mutations in *IDH2*, MIM# 613657) in 2010, it was retrospectively established that the two studies from 1999 consisted of heterogeneous groups including both types of D-2-HGA patients, as well as combined D,L-2-HGA patients, which resulted in a heterogeneous clinical description of mild and severe phenotypes.

Here, we present novel clinical data of fourteen D-2-HGA type I and nineteen D-2-HGA type II patients. The data were collected using clinical questionnaires and the most frequent clinical findings are summarized in this *Review*. The age of onset of D-2-HGA type I patients is within the first six years of life, whereas for D-2-HGA type II the age of onset was within two years (Table 1). The most frequently observed signs in D-2-HGA are developmental delay, hypotonia and seizures for both type I and type II, but seizures were observed with a much higher frequency in the type II group (Table 1). All D-2-HGA type II patients had developmental delay. This delay was also more severe than in D-2-HGA type I patients. In 9 out of 19 D-2-HGA type II patients cardiomyopathy (most often dilated, in one case hypertrophic) was observed, which was completely absent in the D-2-HGA type I population. The disease course of D-2-HGA is most often progressive, ranging from mild to severe, but a few individuals were reported to have a static disease or even improve in their overall functioning. Life expectancy for D-2-HGA type II is variable ranging from several months up the early adulthood (Table 1). For D-2-HGA type I life expectancy is uncertain due to the

limited data; one patient deceased in his third week of life and was diagnosed with necrotizing enterocolitis upon post mortem examination, whereas three others are currently 5, 7 and 12 years old.

Table 1: Clinical observations in D-2-HGA type I and type II

	Symptoms	**D-2-HGA type I**	**D-2-HGA type II**
Number of patients		14	19
Age onset (yr)	Mean	1	0.25
	Range	0-6	0-2
Signs during disease (% of type I or type II)	Developmental delay	78 (11 pts.) *- 3 pts. unaffected* *- 5 pts. mild* *- 3 pts. moderate* *- 3 pts. severe*	100 (19 pts.) *- 0 pts. unaffected* *- 2 pts. mild* *- 6 pts. moderate* *- 11 pts. severe*
	Hypotonia	57 (8 pts.)	89 (17 pts.)
	Seizures	29 (4 pts.)	79 (15 pts.)
	Cardiomyopathy	0	47 (9 pts.) *- 7 pts. dilated* *- 1 pts. hypertrophic* *- 1 pts. unknown*
Alive (yr)	Mean age	8 (n=3)	8.4 (n=10)
	Range	5, 7, 12	2.8-19
Died (yr)	Mean age	3 weeks* (n=1)	6.5 (n=9)
	Range	-	0.3-22
Unknown		n=10	-

***** diagnosed necrotizing enterocolitis in post mortem examination

Currently, 95 patients with D-2-HGA have been diagnosed (including unpublished patients known in our unit), of whom many were diagnosed before the discovery of the genetic defects (Table 2). Patients originate from various ethnic backgrounds worldwide. Consanguinity of the parents was frequently present in D-2-HGA type I and absent in D-2-HGA type II. For 50% of the population DNA was available for D-2-HGA type I or type II differentiation: 26 type I and 24 type II patients were diagnosed. In two isolated cases no mutations were found in *D2HGDH* or *IDH2* leaving the etiology in these patients unknown. Several patients have previously been reported as D-2-HGA who showed (to lesser extent) increased L-2-HG as well. Six patients were published, who were recognized to have combined D,L-2-HGA, which will be discussed later in detail. Another small group of D-2-HGA patients affected with comorbidity skeletal dysplasia is included in this *Review* in detail later on.

The clinical descriptions of previously published case reports vary largely from unaffected to severely affected with many symptoms similar to the data summarized in Table 1. Several other clinical features have been reported, including macrocephaly, dysmorphic features and cerebral visual impairment. Unfortunately, these patients have not been differentiated for D-2-HGA type I or type II. The present data (Table 1)

show that cardiomyopathy is exclusively found in D-2-HGA type II, but in one D-2-HGA type I case report (homozygous for c.458T>C; p.Met153Thr in *D2HGDH*) an increased cardiothoracic index and hypertrophic cardiomyopathy was diagnosed (Haliloglu et al. 2009).

Table 2: Overview of number of D-2-HGA patients divided in specific groups

D-2-HGA	Remarks	References
Type I (n=26)	1 patient affected with comorbidity of Sanfilippo Syndrome type C (Pervaiz et al. 2011)	Gibson 1993a; Craigen 1994; van der Knaap 1999b; Misra 2005; Struys 2005a,b; Haliloglu 2009; Kranendijk 2010a; Pervaiz 2011
Type II (n=24)	15 published pts. 9 unpublished pts.*	Geerts 1996; Amiel 1999; van der Knaap 1999a,b; Clarke 2003; Kranendijk 2010b
Undifferentiated (n=43)	DNA unavailable for *D2HGDH* and *IDH2* sequencing 18 published pts. 25 unpublished pts.*	Chalmers 1980; Nyhan 1995; Sugita 1995; Baker 1997; Wagner 1998; van der Knaap 1999a,b; Eeg-Olofsson 2000; Kwong 2002; Wang 2003; Mahfoud 2009
Unknown (n=2)	D-2-HGA type I and type II were excluded: no mutations detected in *D2HGDH* or *IDH2*	Kranendijk 2010b
Combined D,L-2-HGA (n=11)	Increased D-2-HG and L-2-HG 6 published pts. 5 unpublished pts.*	Wagner 1998; Amiel 1999; van der Knaap 1999a,b; Muntau 2000; Wajner 2002; Read 2005
Skeletal dysplasia (n=6)	3 published pts. 3 unpublished pts.*	Talkhani 2000; Honey 2003; Bayar 2005

* Unpublished patients are diagnosed and/or archived in our laboratory records.

Remarkable observations were made in 4.5-year-old female monozygotic twins who are affected with D-2-HGA type I (compound heterozygous *D2HGDH*) (Misra et al. 2005). One twin presented with multiple congenital anomalies, severe developmental delay, and abnormal neuroradiological findings, while the other twin reached all of her major motor and language milestones appropriately and her clinical course was generally uncomplicated. The different observations in these monozygotic twins illustrate that postzygotic genetic changes, epigenetic differences, or environmental factors influence phenotypic outcome and progression of this disease.

Neuroimaging was performed in D-2-HGA patients, but those were published before the genetic defects were identified. In these cohorts of undifferentiated D-2-HGA and combined D,L-2-HGA heterogeneous findings were reported, including enlargement of the lateral ventricles, enlarged frontal subarachnoid spaces, subdural effusions, subependymal pseudocysts, signs of delayed cerebral maturation and multifocal cerebral white-matter abnormalities (van der Knaap et al. 1999a; van der Knaap et al. 1999b). MRIs are currently collected to perform an extensive study enrolling genetic and biochemically confirmed D-2-HGA type I and type II patients.

Mutations in D2HGDH cause D-2-HGA type I

In the pursuit of the defect in D-2-HGA, enzymes were isolated from frozen rat liver to screen for a dehydrogenase acting on D-2-HG by applying DEAE-Sepharose chromatography combined with spectrophotometric and radioactive detection (Achouri et al. 2004). The group of Prof. Van Schaftingen identified the gene *D2HGDH* (GeneBank 728294, NM_152783, MIM# 609186) encoding D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) having high specificity for D-2-HG. Subsequent detection of *D2HGDH* mutations in patients confirmed the genetic defect in D-2-HGA (Misra et al. 2005; Struys et al. 2005a; Struys et al. 2005b; Haliloglu et al. 2009; Kranendijk et al. 2010a; Pervaiz et al. 2011). At present, 26 patients have been reported with 31 different mutations in *D2HGDH* spread over the gene (Figure 4). Eleven mutations were predicted to result in truncated enzymes, whereas the remaining twenty missense mutations alter conserved amino acids across species suggesting pathogenicity. In a few cases the pathogenicity was investigated by overexpression studies of the mutated allele and indeed confirmed their pathogenicity (Struys et al. 2005b). Patients carrying pathogenic homozygous or compound heterozygous mutations in *D2HGDH* are denoted D-2-HGA type I (encompass 50% of the D-2-HGA population). The inheritance pattern has an autosomal recessive trade.

Mutations in IDH2 cause D-2-HGA type II

Studies in human cancer genetics revealed increased incidence of heterozygous mutations in isocitrate dehydrogenase 1 and 2 (*IDH1*, *IDH2*), which conferred on the enzymes a new

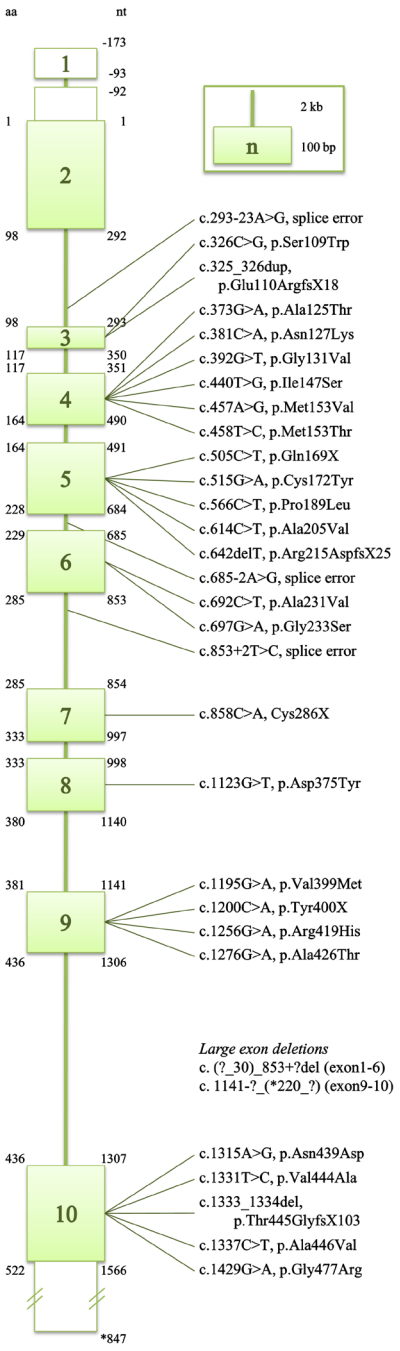

Figure 4: Mutations eported in *D2HGDH* (Misra 2005; Struys 2005a,b; Haliloglu 2009; Kranendijk 2010a; Pervaiz 2011)

function, namely the conversion of 2-KG to D-2-HG leading to abnormal production of the latter (Parsons et al. 2008; Dang et al. 2009; Ward et al. 2010). This finding led us to the discovery of germline heterozygous *IDH2* mutations in D-2-HGA patients (GeneBank 3418, NM_002168, MIM# 147650), which is the second defect observed in D-2-HGA and is denoted D-2-HGA type II (Kranendijk et al. 2010b). Fourteen patients carried the heterozygous c.419G>A, p.Arg140Gln mutation ($IDH2^{wt/R140Q}$); only one patient was heterozygous for c.418C>G, p.Arg140Gly ($IDH2^{wt/R140G}$). In DNA of 24 patients the gain-of-function mutation has been detected encompassing 50% of the D-2-HGA population (Table 2). The *IDH2* mutations were not detected in eight out of nine sets of parents, indicating that the heterozygous mutations arose *de novo* and that D-2-HGA type II is an autosomal dominant trait. However, in one family three subsequent affected pregnancies were diagnosed suggesting germline mosaicism in the mother who was confirmed to have somatic mosaicism in her blood.

Metabolism in D-2-HGA

Different mechanisms form the basis of D-2-HG accumulation in D-2-HGA type I and type II. HOT enzymes naturally convert 2-KG to D-2-HG (Figure 5) (Struys et al. 2005c), of which the latter has no known physiological function in human metabolism. To prevent loss of valuable carbon moieties, as well as to protect against potential intoxication, D-2-HG is converted back to 2-KG via D-2-hydroxyglutarate dehydrogenase (D-2-HGDH, EC 1.1.99.-). Impaired D-2-HGDH enzyme activity was detected in lymphoblasts and fibroblasts derived from D-2-HGA type I patients, whereas normal activities were found in D-2-HGA type II (Table 3) (Wickenhagen et al. 2009; Kranendijk et al. 2010a). Consequently, D-2-HG accumulation in D-2-HGA type I is caused by an impaired D-2-HGDH activity.

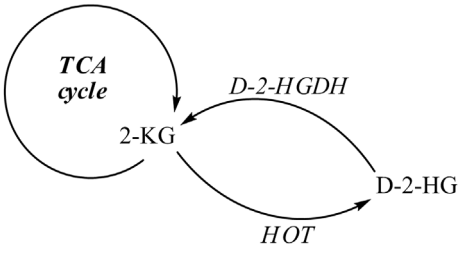

Figure 5: D-2-HG is formed from 2-KG via HOT. D-2-HGDH catalyzes the conversion of D-2-HG to 2-KG. D-2-HG accumulates in D-2-HGA type I patients when D-2-HGDH is impaired.

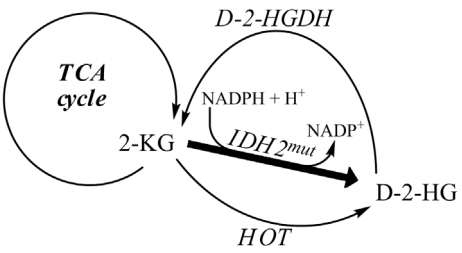

Figure 6: Novel IDH2-mutant gain-of-function produces D-2-HG from 2-KG in addition to production of D-2-HG via HOT. It is hypothesized that D-2-HGDH cannot fully metabolize all of the generated D-2-HG, resulting in D-2-HG accumulation in D-2-HGA type II

In D-2-HGA type II heterozygous mutations were found in isocitrate dehydrogenase 2 (IDH2, EC 1.1.1.42) at residue 140 changing the amino acid arginine to glutamine or glycine (Kranendijk et al. 2010b). Wild type IDH2 reversibly catalyzes the reaction of isocitrate to 2-KG using the NADP(H) couple, whereas the IDH2$^{wt/R140Q}$-mutant gained a new function converting 2-KG to D-2-HG using NADPH as electron donor (Figure 6). Indeed, in lymphoblasts obtained from D-2-HGA type II an eight fold increase of IDH2$^{wt/R140Q}$ reaction rate was observed as compared to controls and D-2-HGA type I (Table 3)(Kranendijk et al. 2011). Apparently, the capacity of the well functioning D-2-HGDH is insufficient to deal with the excess of D-2-HG formed by IDH2$^{wt/R140Q}$.

Table 3: Enzyme activities of D-2-HGDH and IDH2$^{wt/R140Q}$ in D-2-HGA cell lines

	D-2-HGDH fib. (pmol h^{-1} mg prot.$^{-1}$)	**D-2-HGDH lyb.** (pmol h^{-1} mg prot.$^{-1}$)	**IDH2$^{wt/R140Q}$ lyb.** (pmol h^{-1} mg prot.$^{-1}$)
	mean (n); range	mean (n); range	mean (n); range
Controls	*456 (5); 247-665*	*1409 (5); 273-2545*	*1800 (5); 1400-2100*
D-2-HGA type I	22 (5); 0-41	12 (2); 2, 21	1900 (2); 1900, 1900
D-2-HGA type II	338 (14); 204-634	1062 (4); 570-1503	14400 (5); 12000-18800

fib.=fibroblasts; lyb.=lymphoblasts (Wickenhagen 2009; Kranendijk 2010a, 2011)

Table 4: D-2-HG concentration in body fluids and lymphoblasts of D-2-HGA patients

	Urine (mmol/mol creat.)	**Plasma** (µmol/L)	**CSF** (µmol/L)	**Lymphoblasts** (nmol/mg prot.)
	mean (n); range	mean (n); range	mean (n); range	mean (n); range
Controls	*6 (18);2.8-17*	*0.7 (10); 0.3-0.9*	*0.1 (10); 0.07-0.3*	*0.11 (5); 0.06-0.13*
D-2-HGA type I	969 (20);103-2414	68 (7); 26-123	13 (3); 6-18	1.8 (2); 1.7-1.9
D-2-HGA type II	2486 (19);448-11305	366 (9); 99-757	79 (4); 30-172	15.1 (5); 1.9-28.5

(Gibson 1993b; Kranendijk 2010a,b, 2011)

The different mechanisms causing D-2-HGA type I and type II are reflected in the D-2-HG concentrations observed in urine, plasma, CSF and intracellularly in cultured lymphoblasts (Table 4) (Kranendijk et al. 2010a; 2010b; 2011). In all matrices highly increased D-2-HG concentrations were detected in D-2-HGA, whereas in D-2-HGA type II the concentrations were 2 to 8 times higher than in D-2-HGA type I. Concentrations in plasma are higher than in CSF in both patient groups. Extensive metabolic screening (*e.g.* organic acids, amino acids, acylcarnitines, GABA) was performed, but the only consisting biochemical marker remained D-2-HG in these diseases, stressing that the chiral counterpart L-2-hydroxyglutaric acid (L-2-HG) is always within normal limits.

Pathophysiological consequences in D-2-HGA

D-2-HGA type I and type II are genetically unrelated. Nevertheless, they share the biochemical hallmark of D-2-HG accumulation across all body fluids and share the same clinical signs during disease, apart from cardiomyopathy. This suggests a strong

correlation of severely increased D-2-HG levels with developmental delay, hypotonia and seizures in D-2-HGA.

D-2-HGDH and IDH2 are mitochondrial enzymes. Consequently, the origin of D-2-HG accumulation is located in the mitochondria potentially disrupting local processes. Intracellular/mitochondrial D-2-HG concentrations are not known, but plasma concentrations are often used as an alternative measure. D-2-HG concentrations in plasma are 30 (26 μM) to 840 (757 μM) times increased in patients with D-2-HGA (Table 4). In D-2-HGA type II the mean plasma levels are ~5 times higher than in D-2-HGA type I with little overlap in the ranges. The frequency and severity of developmental delay, hypotonia and seizures observed in D-2-HGA type II are (slightly) higher than in D-2-HGA type I (Table 1), therefore it is tempting to speculate that there is a correlation between increasing D-2-HG concentrations and increasing frequency and/or severity of the clinical findings.

Tissues from rat and chick were exposed *in vitro* to increasing D-2-HG concentrations and downregulated the creatine kinase, complex IV and complex V enzymes (da Silva et al. 2002; Kolker et al. 2002; da Silva et al. 2003a; da Silva et al. 2003b; da Silva et al. 2004). Induced oxidative stress and markedly impaired mitochondrial energy metabolism were correlated with increased D-2-HG concentrations (Kolker et al. 2002; Latini et al. 2003b; Latini et al. 2005). Increased synaptosomal glutamate uptake was observed with increased D-2-HG as well, without effecting other synaptosomal parameters studied, providing no support for a direct excitotoxic action for D-2-HG (Junqueira et al. 2004). In contrast with this, Kolker et al. did find that D-2-HG induced excitotoxic cell damage in primary neuronal cultures involving NMDA receptor activation (Kolker et al. 2002). Although all these studies showed effects of D-2-HG on various processes which are likely related to neurodegeneration, the mechanisms explaining the phenotype are still poorly understood.

In our cohort of 33 D-2-HGA patients, cardiomyopathy was only found in D-2-HGA type II, which suggests a strong correlation with the $IDH2^{wt/R140Q}$ gain-of-function mutation and not only with increased D-2-HG concentration. The large amounts of D-2-HG accumulation are facilitated by the consumption of large amounts of 2-KG and NADPH, hypothetically reducing mitochondrial and cellular 2-KG and NADPH concentrations. Subsequent research should focus on the confirmation of this hypothesis and search for causal consequences in physiology. This $IDH2^{wt/R140Q}$ gain-of-function received much attention in cancer research, since it was first discovered in brain tumors (Parsons et al. 2008). Although, D-2-HGA patients have a shortened lifespan and therefore it cannot be excluded that patients do not live long enough to develop cancer, neoplastic disorders have not been reported in D-2-HGA type I or type

II. These patients have highly elevated D-2-HG throughout their whole body, therefore it is unlikely that D-2-HG contributes to the development of cancer, which rebuts the speculation that D-2-HG is an onco-metabolite (Dang et al. 2009; Ward et al. 2010).

Nevertheless, it was found that the hypoxia-inducible factor subunit-1α (HIF-1α), a transcription factor that facilitates tumor growth when oxygen is low and whose stability is regulated by 2-KG, was higher in human gliomas harboring an *IDH1* mutation than in tumors without a mutation (Zhao et al. 2009). Xu et al. reported that D-2-HG functions as a competitive inhibitor of multiple 2-KG dependent dioxygenases which resulted in genome-wide change of histone and DNA methylation, hypothetically contributing to tumorigenesis through altering epigenetic control and potentially changing the differentiation of stem cells (Xu et al. 2011). This finding fits with the observation in the monozygotic twins affected with D-2-HGA type I: they start off with identical genes, but develop a different phenotype (Misra et al. 2005). The discovery of an identical mechanism causing D-2-HG accumulation in D-2-HGA type II and cancer link these diseases with each other and may provide further insights in pathophysiology (Bhagwat et al. 2010).

Therapeutic perspectives

Presently, strategies for therapeutic interventions in D-2-HGA are absent, apart from control of seizures with anti-epileptic drugs. The etiology of D-2-HGA type I was found in the impaired enzyme D-2-HGDH unable to convert D-2-HG to 2-KG causing D-2-HG accumulation (Figure 5). No residual enzyme activity was reported which could be increased by stimulating co-substrates/factors. D-2-HGDH catalytic activity was found being unaffected by FAD, however this does not exclude the possibility that the enzyme has tightly bound FAD (Achouri et al. 2004). D-2-HGDH corresponds genetically to the homologous FAD-dependent D-lactate dehydrogenase and therefore positive effects of riboflavin therapy cannot be excluded. On the other hand, D-2-HG production runs via HOT enzymes (Figure 3), but inhibiting HOT will cause probably much side effects disrupting GHB and SSA levels. Ideally, introduction of a functioning D-2-HGDH enzyme would be favored, but that is a perspective for future technologies.

In D-2-HGA type II *IDH2* gain-of-function mutations result in D-2-HG overproduction. Inhibiting IDH2-mutants with oxaloacetate resulted in decreasing D-2-HG production, a potential therapeutic strategy (Kranendijk et al. 2011). Development of a highly specific drug which (covalently) binds to mutated IDH2-subunits, but does not interact with wild type IDH2, could ameliorate the disease causing defect. Consequently, D-2-HG levels will normalize and the vast amounts of 2-KG and NADPH consumed by IDH2-mutants will remain present.

L-2-hydroxyglutaric aciduria

L-2-hydroxyglutaric aciduria (L-2-HGA, MIM# 236792) is a rare, inherited metabolic encephalopathy with an autosomal recessive trade of inheritance. Today, the genetic defect is well understood, as well as the primary enzymatic impairment and metabolic mechanisms which appointed L-2-HGA as a "disorder of metabolite repair". However, the pathophysiology leading to white matter abnormalities in the brain are poorly understood.

Clinical manifestations in L-2-HGA

Since the first L-2-HGA case report (Duran et al. 1980) additional extensive clinical descriptions were published (Barth et al. 1993; Barbot et al. 1997; Achouri et al. 2004; Topcu et al. 2005; Steenweg et al. 2009; Steenweg et al. 2010). A rather homogeneous clinical picture was described by all groups, summarized in Table 5. The studies performed by Steenweg and co-workers were the most extensive ones that described 106 cases.

Table 5: Clinical observations in L-2-HGA reported in literature

	Barth 1992, 1993	**Barbot 1997**	**Topcu 2005**	**Steenweg 2009, 2010**
Number of patients - clinical description	12	6	29	106
Age onset	After infancy	½-2 yr (100%)	1-10 yr (67%) 11-18 yr (24%) 19-30 yr (7%)	0-7 yr (97%)
Insidious onset	+	+		+
Signs at diagnosis				
developmental delay	+ (33%)	+ (83%)	+	+ (52%)
epilepsy	+ (1 pt.)	+ (1 pt.)		+ (42%)
cerebellar ataxia	+ (1 pt.)		+	+ (20%)
Signs during disease course				
developmental delay	+ (100%)	+ (100%)	+ (79%)	+ (93%)
cerebellar ataxia	+ (92%)	+ (100%)	+ (66%)	+ (82%)
epilepsy	+ (50%)	+ (67%)	+ (41%)	+ (72%)
macrocephaly			+ (52%)	+ (48%)
extrapyramidal symptoms	+ (33%)			+ (38%)
Progression	Chronic, slowly	Slowly	Static	Slowly
Number of patients - MRI performed	10	6	24	56
Highly characteristic MRI abnormalities*	+	+	+	+

* Combination of predominantly subcortical cerebral white matter abnormalities and abnormalities of the dentate nucleus, globus pallidus, putamen, and caudate nucleus.

An insidious onset of the disease starting in childhood was observed by most researchers. Developmental delay, epilepsy and cerebellar ataxia are the predominant signs seen at diagnosis. The disease course is slowly progressive without fluctuations or acute deteriorations. As a result, L-2-HGA patients with only mild symptoms are often diagnosed as teenager, or sometimes even as adults, but retrospectively an earlier onset in childhood is recognized in most patients. Virtually all patients display a mental and motor developmental delay, and cerebellar dysfunction. Epilepsy is diagnosed in roughly two-third of the patients. Macrocephaly and extrapyramidal symptoms including tremor and dystonia are observed in less than half of the patients. Steenweg et al. mentioned that the hypotonia and spasticity observed in their cohort tended to be dependent on disease duration, with hypotonia in the earlier stages and spasticity in the later stages. Loss of milestones, like walking without support and development of speech difficulties, was reported as well.

A highly characteristic pattern of MRI abnormalities was identified in L-2-HGA. Steenweg et al. systematically evaluated the MRIs of 56 patients in correlation with the disease duration (Steenweg et al. 2009). They found a combination of predominantly subcortical cerebral white matter abnormalities and abnormalities of the dentate nucleus, globus pallidus, putamen, and caudate nucleus. These results were in close agreement with previous descriptions in the literature (Table 5) (Barth et al. 1993; Barbot et al. 1997; Topcu et al. 2005). With the progression of the disease, white matter and basal ganglia signal intensity abnormalities became more diffuse, followed by cerebral white matter atrophy. Barbot et al. found a strong correlation between the severity of the clinical manifestations and the extent of the lesions on MRI in a limited cohort of 6 patients. Steenweg et al. did not report this correlation in their large cohort.

As mentioned, L-2-HGA is a slowly progressive disease and acute metabolic derangements have not been reported. However, rapid deteriorations have been reported in nine cases, which were related to complications caused by the presence of brain tumors of various pathology (Moroni et al. 2004; Haliloglu et al. 2008; Aghili et al. 2009). Additionally, Vilarinho et al. reported two children and one adult with brain astrocytomas in their series of 21 L-2-HGA patients of Portuguese origin (Vilarinho et al. 2005), whereas Steenweg et al. mentioned one patient out of 56 affected with a brain tumor in the cerebral cortex (Steenweg et al. 2009). These 13 cases of L-2-HGA patients with brain tumors suggest a potential association of L-2-HGA with CNS tumors. The authors describing these patients speculate about several possible mechanisms and suggest that the metabolite L-2-HG itself may predispose to brain oncogenesis. Until now, the pathophysiology of tumorigenesis in L-2-HGA is unknown and further research into metabolic pathways and genetic defects is needed.

Remarkably, two extracranial tumors were diagnosed in L-2-HGA: one case had a bone tumor involving the right frontal region of the calvaria (Larnaout et al. 2007), whereas another L-2-HGA patient had a Wilms tumor (nephroblastoma) (Rogers et al. 2010). These two isolated findings of non-CNS tumors in L-2-HGA may be a coincidence, a subsequent study in 21 Wilms tumor tissues did not reveal increased L-2-HG levels (Rakheja et al. 2011).

Mutations in L2HGDH

Twenty-four years after the description of the first L-2-HGA patient, two research groups identified independently with different strategies and techniques the gene causing L-2-HGA. Rzem et al. applied a similar strategy to identify the gene causing L-2-HGA as they had used for the identification of *D2HGDH* in D-2-HGA type I (Rzem et al. 2004). They purified an enzyme from frozen rat liver by chromatography which acted as a dehydrogenase converting L-2-HG to 2-KG. Using spectrophotometric and radioactive assays several properties of the proposed L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) were determined which led via database searches to the identification of the gene *L2HGDH* (GeneBank 79944, NM_024884, MIM# 609584). Subsequently, homozygosity mapping of three unrelated consanguineous families with L-2-HGA patients confirmed the link between the *L2HGDH* gene and L-2-hydroxyglutaric aciduria. In another independent homozygosity mapping study performed by Topçu et al. the gene *C14orf160* was identified which carried mutations in 21 biochemically and clinically confirmed L-2-HGA patients from 15 mostly consanguineous Turkish families (Topcu et al. 2004).

Since the identification of the genetic defect in L-2-HGA, mutations in *L2HGDH* have been reported worldwide, recently summarized in a *Mutation Update* (Steenweg et al. 2010) and registered in the *Leiden Open Variation Database* (LOVD, last update 30-May-2011) (www.LOVD.nl/L2HGDH 2011). Presently, 86 unique variants are described and registered in 164 individuals who are homozygous/compound heterozygous (157 index, 7 siblings). The majority of the variants concerns missense mutations in the coding region of the gene. Less than 10% of the variants are located in the introns. The missense mutations usually involve highly conserved amino acids predicting impaired L-2-HGDH enzyme activity.

Metabolism in L-2-HGA

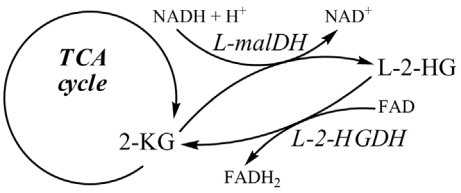

Figure 7: L-2-HG is formed via the non-specific interaction of L-malDH with 2-KG using NADH. The "enzyme of metabolite repair" L-2-HGDH catalyzes the inter-conversion of L-2-HG to 2-KG using FAD as cofactor. L-2-HG accumulates in L-2-HGA patients when L-2-HGDH is impaired.

The concurrent discovery of the gene *L2HGDH* and the function of L-2-hydroxyglutarate dehydrogenase (L-2-HGDH, EC 1.1.99.2) solved the enigma of the supraphysiological L-2-HG concentrations observed in L-2-HGA. As mentioned before, L-2-HG is formed from the non-specific conversion of 2-KG by L-malate dehydrogenase (L-malDH), which uses NADH as the electron and proton donor for the reaction (Figure 7) (Rzem et al. 2007). This reaction is presumed to be an "unwanted" side effect of the L-malate to oxaloacetate reaction catalyzed by L-malDH in the TCA cycle. The normal function of L-2-HGDH is to "repair" this metabolic imperfection, since L-2-HG has no known physiological function in human. To prevent loss of valuable carbon moieties in the metabolic system, as well as to protect for potential toxic effects, L-2-HG is converted back to 2-KG via L-2-HGDH using FAD as cofactor. Mutations found in *L2HGDH* predict an impaired L-2-HGDH enzyme being responsible for the L-2-HG accumulation in body fluids. The above described mechanism led Rzem and co-workers to appoint L-2-HGA a "disorder of metabolite repair".

Transfection studies performed in human embryonic kidney cells overexpressing human *L2HGDH* confirmed the loss of L-2-HGDH enzyme activity when mutations were incorporated (Rzem et al. 2006). The relationship between molecular (mutations in *L2HGDH*) and biochemical observations (increased L-2-HG) in L-2-HGA patients was unambiguously affirmed with a highly specific L-2-HGDH enzyme assay applying stable isotope labeled L-[3,3,4,4-$^2H_4$]2-hydroxyglutaric acid and LC-MS/MS (Kranendijk et al. 2009). Severely impaired L-2-HGDH enzyme activity was detected in fibroblasts, lymphoblasts and lymphocytes obtained from 15 out of 15 patients affected with L-2-HGA. Only in one patient residual activity (20% of the mean L-2-HGDH activity of controls) was detected and this patient was compound heterozygous for two missense mutations: a relatively mild type of mutation which may not disable completely the catalytic property.

Markedly increased L-2-HG excretion is the biochemical hallmark of L-2-HGA (Table 6). Compared to controls, urinary L-2-HG is 10 to 300 times increased, but correlations with the severity of the disease were not found (Barbot et al. 1997;

Steenweg et al. 2010). However, when *L2HGDH* variants were correlated with urinary L-2-HG concentrations, significant differences were observed. Patients with two missense mutations excreted 25-50% less L-2-HG than patients with two presumed null mutations (Table 6), which suggests the presence of residual enzyme activity in patients with missense mutations (Steenweg et al. 2010). L-2-HG levels in plasma and CSF are elevated as well, whereas the increased CSF/plasma ratio indicates an increased metabolism in the brain towards L-2-HG production (Table 7).

Table 6: Urinary L-2-HG concentrations for reported patients and controls

L-2-HGA patients (n)	**Urinary L-2-HG** (mmol/mol creat.)	**References**
Controls (18)	*1.3- 19*	*Gibson 1993b*
L-2-HGA (9)	332-2742	Gibson 1993b
L-2-HGA (12)	226-4299	Barth 1993
L-2-HGA (7)	630-1420	Barbot 1997
L-2-HGA (29)	1000-5520	Topcu 2005
L-2-HGA (15)	671-3392	Kranendijk 2009
L-2-HGA (106)	350-3357	Steenweg 2010
L-2-HGA (mutations L2HGDH)		Steenweg 2010
c.905C>T (6)	1090 p=0.012	
c.530_533delinsATT (9)	2147	
L-2-HGA (mutations L2HGDH)		Steenweg 2010
missense mutations (28)	1431 p=0.012	
presumed null mutations (28)	1916	

Lysine is moderately elevated in CSF and plasma (Table 7). Barbot et al. diagnosed mildly elevated urinary lysine concentrations in 4 out of 6 patients, but Barth et al. observed no abnormalities in urinary lysine (Barth et al. 1993; Barbot et al. 1997). A direct relation with the increased L-2-HG levels is not made, but the data showing higher lysine levels in CSF compared to plasma suggest that the lysine accumulation, equally to L-2-HG, has its origin in the brain. Kamoun et al. pointed out that hyperlysinemia (MIM #238700) is found in metabolic disorders in which a low 2-KG is observed (Kamoun et al. 2002). The ε-amino group of lysine is transferred via transamination to 2-KG through the intermediate saccharopine to form 2-aminoadipic semialdehyde and glutamic acid, a two step mitochondrial process involving alpha-aminoadipic semialdehyde synthase (AASS). Therefore, the authors suggest that in L-2-HGA the lysine accumulation partly reflects a low mitochondrial 2-KG level.

Table 7: L-2-HG and lysine concentrations in urine, plasma and CSF for reported patients

mean (n); range	**Urine** (mmol/mol creatinine)		**Plasma** (µmol/L)		**CSF** (µmol/L)	
	Controls	L-2-HGA	*Controls*	L-2-HGA	*Controls*	L-2-HGA
L-2-HG						
Gibson 1993b	*6 (18); 1.3-19*	1283 (9); 332-2742	*0.6 (10); 0.5-1.0*	47 (8); 27-62	*0.7 (10); 0.3-2.3*	62 (6); 34-100
Barth 1993	*<52*	1810 (12); 226-4299	*n.d.*	31 (10); 7-84	*n.d.*	122 (6); 23-474
Barbot 1997	*<15*	1000 (6); 650-1420	-	#	-	#
Mean (n); range		1364 (27); 226-4299		39 (18); 7-84		92 (12); 23-474
Lysine						
Gibson 1993b	-	-	-	-	-	-
Barth 1993	*7-45*	27 (4); 11-42	*120-230*	279 (8); 70-380	*10-25*	79 (6); 66-89
Barbot 1997	*7-58*	89 (6); 36-168	*40-163*	285 (6); 185-396	*14-25*	77 (2); 60, 95

n.d.=not detected; #=abnormal high values were detected in plasma and CSF in one patient, as well as an increased CSF/plasma ratio.

Pathophysiological consequences in L-2-HGA

Exposure of rat brain tissues to increased L-2-HG concentrations was performed *in vitro* to search for altered mechanisms. Similar to the outcomes of D-2-HG exposure, L-2-HG significantly inhibited the creatine kinase activity in rat cerebellum homogenates (da Silva et al. 2003c), induced oxidative stress (Latini et al. 2003a) and increased glutamate uptake in synaptosomes and synaptic vesicles (Junqueira et al. 2003). The authors speculate that these effects could be related to neurodegeneration in L-2-HGA.

Impaired AASS enzyme activity causes hyperlysinemia, a rare metabolic disorder which is considered to be a “nondisease”, since adverse effects could not be attributed to hyperlysinemia and patients generally achieve normal intellectual performance (Dancis et al. 1983; Saudubray et al. 2007). Therefore, a pathophysiological effect of lysine accumulation in L-2-HGA is not feasible.

The highly characteristic pattern of abnormalities on MRI supports the hypothesis that a specific pathophysiologic mechanism exists, directly or indirectly driven by the supraphysiological concentration of L-2-HG. Unfortunately, pathophysiological mechanisms are still poorly understood demanding further research.

Therapeutic perspectives

There is currently no treatment for L-2-HGA patients, but two anecdotic cases have been reported in which improvement of clinical signs was seen. A 40-year-old female with mild spasticity of the limbs, marked dystonia of the neck and arms and mild intellectual delay was diagnosed with L-2-HGA on the basis of increased urinary 2-HG with confirmed L-2-HG variant (82.7% [normal range, 0.6 to 5.9], equals 505 mmol/mol creatinine), increased lysine in CSF (103.5 μmol/L [control 10 to 30]) and a homozygous mutation in *L2HGDH* c.(845G>A), p.(Arg282Gln) (Samuraki et al. 2008). Brain MRI showed diffuse atrophy and leukoencephalopathy involving mainly subcortical white matter. Treatment with 30 mg/day FAD and 900 mg/day levocarnitine chloride was started, resulting in gradually improved tremor and dystonia in a few weeks. Six months later, she could walk smoothly and urinary 2-HG decreased to 40.4% (equals 247 mmol/mol creatine), which is a 50% reduction. Subsequently, the patient had kept the improved gait, elaborate movements of the hands, and the level of intelligence for more than 4 years after starting treatment.

The second patient is a 16-year-old boy with L-2-HGA with developmental delay since infancy, but mutations in *L2HGDH* were not reported. He was not able to walk without support until 3 years of age (Yilmaz 2009). He had impaired language skills and difficulties with hand movements. Impaired cognitive functions had become more noticeable as he grew up. Treatment with 100 mg/day riboflavin (vitamin B2, precursor of FAD) improved his cognitive and motor performances partially in the first week. He became more independent and started working as a shop assistant. Urinary L-2-HG excretion decreased from 5990 mmol/mol creatinine to 1490 mmol/mol creatinine within three months. When riboflavin treatment was interrupted, significant worsening of both clinical symptoms and urinary L-2-HG excretion (6360 mmol/mol creatinine) occurred. After the reinstitution of riboflavin treatment, the patient returned to his previous clinical status in a week. His brain MRIs after two years of riboflavin treatment was unchanged.

A two month unsuccessful therapeutical trial with 200 mg/day riboflavin was performed in a 9-year-old female with L-2-HGA (urinary L-2-HG 1580 mmol/mol creatinine; plasma lysine 359 μM; CSF 2-HG: 51 μM) (Jequier et al. 2008). She progressively developed action tremor, light gait ataxia, dysarthria and moderate mental retardation. Molecular analysis of *L2HGDH* revealed a homozygous splice site mutation c.738+1G>A (IVS6+1G>A). L-2-HG in urine and lysine in plasma were monitored, but no significant reduction was observed. L-2-HG was measured in CSF before and during treatment, without any significant change. These observations

match with the expectation that the splice site mutation results in a fully impaired L-2-HGDH enzyme activity.

The enzyme L-2-HGDH uses FAD as co-substrate (Figure 7) and its enzyme activity increases with increasing FAD concentrations (Rzem et al. 2006). This mechanism was suggested to reduce the L-2-HG accumulation by (partly) restoring the L-2-HGDH enzyme activity. This can only work for "mild" missense mutations in *L2HGDH* as found in the first patient, whereas truncated enzymes cannot be rescued. The presence of residual enzyme activity in L-2-HGDH with "mild" missense mutations is supported with the finding of lower urinary L-2-HG concentrations in these patients than in presumed null mutations (Table 6). One patient, having compound heterozygous missense mutations in *L2HGDH*, was found to have modest residual L-2-HGDH enzyme activity (Kranendijk et al. 2009), but the results of riboflavin therapy investigating the potential clinical improvements with increasing L-2-HGDH activity and decreasing L-2-HG levels has not been reported in this patient.

Although, riboflavin/FAD treatment may improve the clinical phenotype as shown in two isolated cases, it does not restore the metabolic defect and does not reverse the leukoencephalopathy. Unfortunately, no enzyme studies were performed in these cases supporting the hypothesis of rescued L-2-HGDH activity. Ideally, introduction of a functioning L-2-HGDH enzyme would be favored, as discussed for D-2-HGA type I, but extended studies with more L-2-HGA patients receiving riboflavin therapy may sustain the above described therapeutic intervention.

Combined D,L-2-hydroxyglutaric aciduria

The recognition of this biochemical variant of 2-hydroxyglutaric aciduria was made 20 years after D-2-HGA and L-2-HGA. Currently six cases have been described in literature, of which four were previously classified as D-2-HGA affected with the severe phenotype (Wagner et al. 1998; Amiel et al. 1999; van der Knaap et al. 1999a,b; Muntau et al. 2000; Wajner et al. 2002; Read et al. 2005). Combined D,L-2-hydroxyglutaric aciduria (D,L-2-HGA) is biochemically characterized by moderately increased concentrations of both D-2-HG and L-2-HG in urine, mildly increased in plasma, whereas only D-2-HG is mildly increased in CSF and L-2-HG is normal (Table 8). In all body fluids the D-2-HG concentration is higher than L-2-HG. Lactate and TCA cycle metabolites succinate, fumarate and malate have been reported to be increased occasionally in urine, whereas 2-ketoglutarate was consistently increased in four patients A-D.

Table 8: Biochemical findings in combined D,L-2-HGA patients reported in literature

Patient	**Urine** (mmol/mol creatinine)		**Plasma** (µmol/L)		**CSF** (µmol/L)	
	D-2-HG	L-2-HG	D-2-HG	L-2-HG	D-2-HG	L-2-HG
Control	*2.8-17*	*1.3-19*	*0.3-0.9*	*0.5-1.0*	*0.07-0.3*	*0.3-2.3*
A	315-1185	162-332	2.5	2.3-3.7	2.5	1.2
B	520	142	2.22	1.07	0.49	normal
C*	228	145	--	--	--	--
D*	17.9-1072	25.2-430	2.48	2.22	0.42	normal
E	632, 685, 786	32, 76, 83	--	--	--	--
F	162, 306	127, 152	1.8, 4.6	1.91, 1.7	--	--
Mean (n); range	496 (6); 228-750	161 (6); 64-247	2.6 (4); 2.2-3.2	2.0 (4); 1.1-3.0	1.1 (3); 0.42-2.5	normal (3)

* Siblings; References: *Controls*-(van der Knaap 1999a); A-(Wagner 1998) case2, (van der Knaap 1999b) pat.8, (Muntau 2000) pat.3; B-(Amiel 1999) case2, (van der Knaap 1999a) pat.4; C-(Muntau 2000) pat.1, (van der Knaap 1999a) pat.1; D-(Muntau 2000) pat.2, (van der Knaap 1999a) pat.2; E-(Wajner 2002); F-(Read 2005)

Five patients (A-D, F) showed a rather homogenous clinical phenotype presenting with a severe neonatal epileptic encephalopathy as the main characteristic, often accompanied by respiratory insufficiency resulting in a need for artificial ventilation (Table 9). Four probands died within their first year of life, one deceased at 3.5 years of age. The MRI of these patients showed brain abnormalities encompassing enlarged ventricles, subependymal pseudocysts and delayed gyration and myelination.

Other clinical findings were observed in patient E showing hypotonia, developmental delay, seizures, cardiomyopathy and respiratory distress, whereas the MRI revealed a pattern suggestive of a mitochondrial disease (Wajner et al. 2002). This patient died because of a cardiogenic shock at the age of 10 months. Although, the clinical findings show similarities with D-2-HGA type II, the increased L-2-HG concentration and the

observed MRI abnormalities deviate from D-2-HGA type II. Consequently, we do not consider patient E to be affected with "classic" D,L-2-HGA or D-2-HGA type II and therefore we speculate that patient E has a different etiology than the other five patients.

Table 9: Clinical observations in D,L-2-HGA patients reported in literature

	Patient A	**Patient B**	**Patient C***	**Patient D***	**Patient E**	**Patient F**
Gender	Female	Female	Male	Female	Male	Female
Age at death	8 months	<11 months	3.5 years	2.5 months	10 months	1 month
Consanguinity	-	-	+	+	-	-
Epileptic encephalopathy	+	+	+	+	+	+
Developenmental delay	Severe	Severe	Severe	Severe	Severe	Severe
Respiratory insufficiency	+		+	+	+	
Other signs		Facial dysmorphism			Hypotonia, cardiomyopathy	
MRI abnormalities	+	+	+	Not performed	Suggestive of mitochondrial disease	+
Enlarged ventricles	+	+	+			+
Subependymal pseudocysts	+	+	+			+
Delayed gyration and myelination	+		+		+	+
References	Wagner 1998 Muntau 2000	Amiel 1999	Muntau 2000	Muntau 2000	Wajner 2002	Read 2005

* Siblings

High 2-KG levels in D,L-2-HGA cause probably elevated formation of D-2-HG and L-2-HG via HOT and L-malDH (Figure 3). The extent of increased D-2-HG and L-2-HG concentrations is much lower compared to D-2-HGA and L-2-HGA, indicating a secondary mechanism resulting in the accumulation of D-2-HG and L-2-HG and therefore probably do not provoke this disease. The concurrent findings of increased lactate and TCA cycle metabolites 2-KG, succinate, fumarate and malate are suggestive of a mitochondrial disorder.

DNA of patient B and F was assessed for molecular studies but no mutations were found in *D2HGDH*, *L2HGDH*, or *IDH* (unpublished data). Although, the pattern of inheritance is uncertain, an autosomal recessive trait is probably present, because in the family with consanguineous Caucasian parents two siblings were affected (patient C and D). Because patients A-D and F display biochemical and clinical similarities, exome sequencing of their DNA could probably reveal the genetic defect underlying D,L-2-HGA. Currently, treatment options are not available, but discovery of the defect may initiate therapeutic exploration.

2-hydroxyglutaric aciduria in other disorders

Neoplastic disorders with IDH mutations accumulate D-2-HG

Studies in human cancer genetics revealed recurrent mutations in *isocitrate dehydrogenase 1* and *2* (*IDH1*, *IDH2*), which conferred on the enzymes a new function, namely the conversion of 2-KG to D-2-HG (Parsons et al. 2008; Dang et al. 2009; Ward et al. 2010), which is the identical mechanism causing D-2-HG accumulation in D-2-HGA type II. Consequently, increased D-2-HG levels were detected in samples obtained from malignant gliomas (Dang et al. 2009) and tumor tissues, cells and serum obtained from acute myeloid leukemia patients (Ward et al. 2010; Gross et al. 2010; Sellner et al. 2010) which all had mutations in either *IDH1* or *IDH2*. Whether IDH1 or IDH2 mutations in cancer are a positive or negative prognostic marker depends highly on the pathology of the cancer (Parsons et al. 2008; Yan et al. 2009; Paschka et al. 2010). D-2-HG is considered an onco-metabolite but indisputable evidence has not been presented proving carcinogenic toxicity of D-2-HG.

Metaphyseal chondromatosis with D-2-HGA (MC-HGA, MIM# 271550)

Several patients had increased D-2-HG excretion, but had a remarkably different phenotype of skeletal dysplasia, which has not been reported in D-2-HGA type I and type II. Talkhani et al. published the first patient who was diagnosed with spondyloenchondrodysplasia with concurrent elevated D-2-HG levels in urine (9089 and 8909 mmol/mol creatinine) and plasma (138 and 159 μmol/L), whereas L-2-HG was normal (Talkhani et al. 2000). This girl was diagnosed at the age of 12 months having a severe crippling form of skeletal dysplasia and global developmental delay, although development was slow and continued to improve in her second year of life.

The second publication reports a male infant who was diagnosed at 6 months with skeletal dysplasia, his motor and mental milestones were slightly delayed and metabolic urine screening revealed increased D-2-HG (Honey et al. 2003). At 11 months he was hypotonic, fine motor function was delayed, his speech development was severely delayed but spondyloenchondrodysplasia was the central clinical problem. MRI of the brain demonstrated a number of abnormalities, including moderate dilatation of the lateral ventricles, a large cavum septi pellucidi and cavum Vergae, but white matter maturation was normal.

A third case report described a 17-month-old boy with waddling gait and swollen joints. His motor and mental development, other than delay in walking, was normal and remained normal at 3 years of age (Bayar et al. 2005). Bone survey of the patient revealed severe metaphyseal widening, splaying, cupping and fragmentation. In urinary organic acids screening increased 2-HG was detected in two independent samples (413, 367 mmol/mol creatinine), chiral differentiation confirmed the D-form.

Very recently, Vissers and co-workers detected in four patients affected with Metaphyseal Chondromatosis (MC-HGA), including the patient reported by Bayar et al., somatic mutations of IDH1 (p.Arg132) using exome sequencing of blood DNA (Vissers et al. 2011). These somatic mutations in IDH1 may explain all features of MC-HGA, including sporadic occurrence, metaphyseal disorganization and chondromatosis, urinary excretion of D-2-hydroxy-glutaric acid, and reduced cerebral myelinization.

Multiple Acyl-CoA Dehydrogenase Deficiency (MADD, MIM# 231680)

MADD, also referred as glutaric aciduria type II, is an autosomal recessively inherited disorder of fatty acid, amino acid, and choline metabolism which can result from defects in two flavoproteins: electron transfer flavoprotein (ETF) or ETF:ubiquinone oxidoreductase (ETF:QO) (Olsen et al. 2007; Liang et al. 2009). ETF receives electrons from several dehydrogenases transferring them to ETF-QO which subsequently passes the electrons to ubiquinone in the respiratory chain. Large excretion of glutaric acid can be the biochemical hallmark, but in addition to several other increased organic acids D-2-HG is often observed in organic acid screening. Consequently, it is speculated that D-2-HGDH activity is impaired, since being a dehydrogenase it cannot dispose its electrons to a deficient ETF or ETF:QO.

Succinic semialdehyde dehydrogenase deficiency (SSADH, MIM# 271980)

SSADH deficiency is an autosomal recessively inherited disorder caused by mutations in the *SSADH* gene (Jakobs et al. 1993; Akaboshi et al. 2003). SSADH catalyzes the conversion of succinic semialdehyde (SSA) to succinic acid (SA), of which SSA accumulates when SSADH activity is impaired. Consequently, 4-hydroxybutyrate (GHB) is produced in high amounts via SSA-reductase. Increased GHB in body fluids is the biochemical hallmark of this disease. Additionally, several other metabolites are increased including D-2-hydroxyglutarate, which is believed to be formed via HOT due to the increased concentrations of GHB (Figure 3) (Struys et al. 2006).

Miscellaneous disorders related with 2-HG

Several other disorders show (secondary) increased levels of 2-HG (undifferentiated for D-2-HG or L-2-HG, or combined accumulation of both), often caused by primarily accumulation of 2-KG. For example, DOOR-syndrome is an autosomal recessive malformation syndrome that variably features elevated 2-KG and 2-HG in urine (James et al. 2007). Another example is dihydrolipoyl dehydrogenase (E3) deficiency which shows increased levels of many organic acids including 2-KG and 2-HG (Kuhara et al. 1983).

Final remarks

Strikingly different characteristics of etiology and phenotype of D-2-HGA type I, D-2-HGA type II and L-2-HGA are described, reflecting the distinct metabolic, enzymatic, genetic and clinical identities of these neurometabolic disorders, whereas the etiology in D,L-2-HGA remains unknown (Table 10).

D-2-HGA type I and type II share the biochemical hallmark of D-2-HG accumulation in body fluids, which correlate probably with the clinical outcomes developmental delay, hypotonia and seizures observed in both disorders. The higher excretion of D-2-HG in D-2-HGA type II is probably expressed in higher frequency and severity of the symptoms. Cardiomyopathy is exclusively found in D-2-HGA type II, supposedly related to decreased (mitochondrial) NADPH and 2-KG levels.

D-2-HGA and L-2-HGA have very different clinical presentations (including MRI abnormalities), pointing towards different pathophysiologic consequences of D-2-HG and L-2-HG accumulation in human which are currently poorly understood.

Although, the disease causing IDH2 mutation of D-2-HGA type II is found frequently in various neoplastic disorders, cancer was not reported in D-2-HGA, in contrast to the finding of brain tumors in L-2-HGA. Therefore, we do not consider D-2-HG an "onco-metabolite", whereas L-2-HG accumulation correlates with increased risk of brain tumors.

The characteristics in D,L-2-HGA deviate widely from D-2-HGA and L-2-HGA, suggesting another unique underlying (metabolic) defect.

Presently, the etiologies of D-2-HGA type I, type II and L-2-HGA are well understood with a clear description of the gene-enzyme-metabolite trait. Therapeutic interventions are still not available apart from anecdotal successful riboflavin therapy in L-2-HGA in two cases. Future research should be focused on finding the pathophysiological mechanisms and developing potential therapeutic strategies.

Table 10: Overview 2-hydroxyglutaric aciduria

	D-2-HGA type I (MIM# 600721)	**D-2-HGA type II** (MIM# 613657)	**L-2-HGA** (MIM# 236792)	**D,L-2-HGA**
Metabolites mean (n); range *(controls)*	Increased D-2-HG	Increased D-2-HG	Increased L-2-HG	Increased D-2-HG and L-2-HG
Urine (mmol/mol creat.)				
D-2-HG *(6 (18); 2.8-17)*	969 (20); 103-2414	2486 (19); 448-11305	normal	496 (6); 228-750
L-2-HG *(6 (18); 1.3-19)*	normal	normal	1364 (27); 226-4299	161 (6); 64-247
Plasma (µmol/L)				
D-2-HG *(0.7 (10); 0.3-0.9)*	68 (7); 26-123	366 (9); 99-757	normal	2.6 (4); 2.2-3.2
L-2-HG *(0.6 (10); 0.5-1.0)*	normal	normal	39 (18); 7-84	2.0 (4); 1.1-3.0
CSF (µmol/L)				
D-2-HG *(0.1 (10); 0.07-0.3)*	13 (3); 6-18	79 (4); 30-172	normal	1.1 (3); 0.42-2.5
L-2-HG *(0.7 (10); 0.3-2.3)*	normal	normal	92 (12); 23-474	normal
Other metabolites	--	--	Lysine increased in plasma and CSF, mostly normal in urine	Inconsistently increased urinary 2-KG, succinate, fumarate and lactate
Enzyme	D-2-hydroxyglutarate dehydrogenase D-2-HGDH EC 1.1.99.-	Isocitrate dehydrogenase 2 IDH2 EC 1.1.1.42	L-2-hydroxyglutarate dehydrogenase L-2-HGDH EC 1.1.99.2	--
Defect mechanism	Impaired activity	Gain-of-function	Impaired activity	
Gene	*D2HGDH* GeneBank 728294 NM_152783 MIM# 609186	*IDH2* GeneBank 3418 NM_002168 MIM# 147650	*L2HGDH* GeneBank 79944 NM_024884 MIM# 609584	--
Type of mutations	Heterogeneous	c.419G>A, R140Q c.418C>G, R140G	Heterogeneous	
Trait	Autosomal recessive	Autosomal dominant	Autosomal recessive	
Clinical signs	Developmental delay Hypotonia Seizures	Developmental delay Hypotonia Seizures Cardiomyopathy	Developmental delay Epilepsy Cerebellar ataxia	Severe neonatal epileptic encephalopathy
	Onset at 0-6 years Lifespan unknown MRI abnormalities	Onset at 0-2 years Shortened lifespan MRI abnormalities	Insidious onset in childhood Highly distinct MRI abnormalities	Onset in infancy Shortened lifespan MRI abnormalities
Therapeutic strategies	--	--	Riboflavin may improve symptoms	--
Cancer	--	Not reported, but IDH2 mutations in neoplastic disorders	Increased incidence of brain tumors	--
Key references	Achouri 2004 Wickenhagen 2009 Struys 2006 Kranendijk 2010a	Kranendijk 2010b Kranendijk 2011	Rzem 2004 Aghili 2009 Kranendijk 2009 Steenweg 2009	Muntau 2000 Read 2005

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Summary and general discussion

2-Hydroxyglutaric acidurias (2-HGA) are inborn errors of metabolism defined by increased physiological 2-hydroxyglutarate concentrations observed in patient's body fluids like urine, plasma and cerebrospinal fluid, expressing neurological impairments which manifest at young age. Originally, two classes were identified based on the chirality of the accumulating metabolite: D-2-hydroxyglutaric aciduria (D-2-HGA) and L-2-hydroxyglutaric aciduria (L-2-HGA) [1,2]. As understanding of these disorders progressed, a third variant was described and defined as combined D-2- and L-2-hydroxyglutaric aciduria (D,L-2-HGA) [3].

The identification of mutations in the gene *L2HGDH* encoding L-2-hydroxyglutarate dehydrogenase (L-2-HGDH) revealed the metabolic defect in L-2-HGA [4,5]. Enzymatic confirmation of impaired L-2-HGDH catalytic activity was performed in patient's fibroblast, lymphoblast and lymphocyte lysates with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (**Chapter 2**). This enzyme assay, based on the conversion of stable isotope labeled L-2-hydroxyglutarate (L-2-HG) to stable isotope labeled 2-ketoglutarate (2-KG), provided a functional link between the metabolic and genetic features of this disorder.

Two anecdotic L-2-HGA case reports described promising results with improved clinical signs and a decrease in urinary L-2-HG concentrations upon oral supplementation of FAD or riboflavin (a precursor of FAD) [6,7]. It is hypothesized that the enzyme's catalytic activity of FAD-dependent L-2-HGDH can be restored by increasing physiological FAD concentrations. The effect of increasing catalytic activity with increasing FAD concentrations was previously shown in a purified enzyme extract of rat liver [4]. In a cohort of eleven L-2-HGA patients only one patient lymphoblast cell lines showed residual L-2-HGDH activity (20% of mean control activity) (**Chapter 2**). Rescuing the enzyme activity with increased physiological FAD concentration is a potential therapeutic strategy which needs further research.

Homogeneous genetic, enzymatic and biochemical characteristics were found in 24 out of 50 D-2-HGA patients (**Chapter 3**). They carried two mutations in the gene *D2HGDH* and impaired D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) catalytic activity was detected. This group of patients was denoted D-2-HGA type I, an autosomal recessive form of D-2-HGA. These patients had significantly lower D-2-hydroxyglutarate (D-2-HG) accumulation in urine, plasma and cerebrospinal fluid compared to D-2-HGA patients without *D2HGDH* mutations.

A novel cause of D-2-HGA was discovered by the identification of *de novo* heterozygous germline mutations in *isocitrate dehydrogenase 2* (*IDH2*) (**Chapter 4**). These patients had no mutations in *D2HGDH*, had normal D-2-HGDH activity and were subsequently denoted D-2-HGA type II, an autosomal dominant form of D-2-HGA. The altered amino acid residue Arg-140 in IDH2-mutants disabled the enzyme's normal ability to convert reversibly isocitrate to 2-KG and conferred on the enzyme a new function: the ability to convert 2-KG to D-2-HG. Significantly higher physiological D-2-HG concentrations were observed in D-2-HGA type II compared to D-2-HGA type I. Several non-related D-2-HGA patients had no mutations in *D2HGDH* and *IDH2*, the defect in these patients remains unresolved.

The presence of IDH2-mutant gain-of-function activity in D-2-HGA type II was confirmed with a functional assay performed in patient lymphoblast lysates (**Chapter 5**). Stable isotope labeled 2-KG is converted to D-2-HG which is detected by LC-MS/MS. Eight fold increased D-2-HG production was detected in D-2-HGA type II compared to controls and D-2-HGA type I. The enzyme D-2-HGDH functions normally in D-2-HGA type II, and apparently D-2-HGDH's capacity is insufficient to convert all D-2-HG produced by the IDH2-mutant. The IDH2-mutant assay was applied for therapeutic exploration by screening endogenous metabolites closely related to 2-KG, D-2-HG or isocitrate for their inhibiting potential. Oxaloacetate appeared to be the most potent competitive inhibitor for decreasing D-2-HG production.

The recognition of D-2-HGA type I and D-2-HGA type II as distinct inborn errors of metabolism was proceeded with the description of the phenotype distilled from data retrieved by international clinical questionnaires (**Chapter 6**). D-2-HGA type I and type II share the biochemical hallmark of D-2-HG accumulation in body fluids, which correlate probably with the clinical features as developmental delay, hypotonia and seizures observed in both disorders. Cardiomyopathy is exclusively found in D-2-HGA type II, supposedly related to decreased (mitochondrial) NADPH and 2-KG levels.

The disease causing IDH gain-of-function mechanism in D-2-HGA type II is found frequently in neoplastic disorders [8,9]. Until now, cancer was not reported in D-2-HGA type I or type II, therefore we do currently not consider D-2-HG an "onco-metabolite" which was suggested by Dang et al. In contrast, increased risk of brain tumors is found in L-2-HGA suggesting carcinogenic properties for L-2-HG. Nevertheless, the discovery of an identical mechanism causing D-2-HG accumulation in D-2-HGA and cancer link these diseases and may provide further insights in pathophysiology.

D-2-HGA and L-2-HGA have very different clinical presentations, pointing towards different pathophysiologic consequences of D-2-HG and L-2-HG accumulation in human. The poorly understood pathophysiologic mechanisms of these disorders need to be explored more intensively to create opportunities for the development of therapeutic strategies.

The (genetic) defect in D,L-2-HGA is still unknown, but homogeneous clinical and metabolic findings in five cases showed similarity suggesting the presence of one disease causing mechanism for this severe neonatal epileptic encephalopathy. Exome sequencing is considered to be a potential strategy for continuing research to find the defect.

Final conclusion

In this *Thesis* it is recognized that D-2-HGA consists of at least two entities: D-2-HGA type I and D-2-HGA type II. This extended the definition to four distinct *neurometabolic disorders* encompassing the majority of diagnosed 2-HGA patients: L-2-HGA, D-2-HGA type I, D-2-HGA type II en D,L-2-HGA (Figure 1). Recently, IDH1 gain-of-function mutations were identified in patients with combined Metaphyseal Chondromatosis and D-2-HGA (MC-HGA) [10], whereas a few patients remain unidentified with a miscellaneous phenotype of unknown origin. The proceedings in metabolic, enzymatic, genetic and clinical studies described in this *Thesis* elucidated D-2-HGA I, D-2-HGA II and L-2-HGA in further detail, and exploration of therapeutic interventions in D-2-HGA type II has been successfully initiated. Resolving the pathophysiological mechanisms in 2-HGA may help to find a proper therapy for these diseases or protect from the adverse effects of D-2-HG and L-2-HG accumulations.

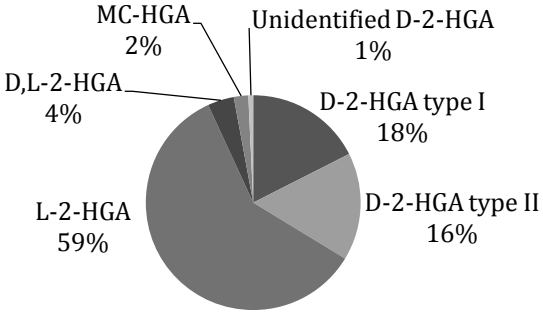

Figure 1: Total 2-hydroxyglutaric aciduria distribution (n=276)

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Nederlandse samenvatting

Kataññutā (Dankbaarheid)

Kataññutā (Gratitude)

Cover description

About the author

Publications

Karakterisering van neurometabole 2-hydroxyglutaaraciduriën en de ontdekking van D-2-hydroxyglutaaracidurie type II

2-Hydroxyglutaaraciduriën (2-HGA) zijn aangeboren stofwisselingsziekten die gekenmerkt worden door fysiologisch verhoogde 2-hydroxyglutaarzuur concentraties in lichaamsvloeistoffen als urine, plasma en liquor. Patiënten vertonen neurologische afwijkingen die zich vaak manifesteren op jonge leeftijd. Oorspronkelijk zijn, gebaseerd op de chiraliteit van de accumulerende metaboliet, twee klassen gedefinieerd: D-2-hydroxyglutaaracidurie (D-2-HGA) and L-2-hydroxyglutaaracidurie (L-2-HGA) [1,2]. Een derde variant is beschreven als "gecombineerde D-2- en L-2-hydroxyglutaaracidurie" (D,L-2-HGA)[3].

L-2-HGA wordt veroorzaakt door mutaties in het gen *L2HGDH* dat codeert voor L-2-hydroxyglutaraat dehydrogenase (L-2-HGDH) [4,5]. De verminderde L-2-HGDH activiteit is gemeten in fibroblasten, lymfoblasten en lymfocyten van patiënten door middel van een methode die gebruik maakt van vloeistof chromatografie – tandem massa spectrometrie (LC-MS/MS) (**Chapter 2**). Deze methode, gebaseerd op de omzetting van stabiel isotoop gelabelde L-2-hydroxyglutaraat (L-2-HG) naar stabiel isotoop gelabelde 2-ketoglutaraat (2-KG), fungeert als de functionele link tussen de metabole en genetische eigenschappen van de aandoening L-2-HGA.

Twee L-2-HGA casussen zijn beschreven waarin therapeutisch oraal toegediend FAD of riboflavine (een precursor van FAD) een verbeterd klinisch beeld liet zien gecombineerd met een verminderde urinaire L-2-HG uitscheiding [6,7]. De katalytische activiteit van het FAD-afhankelijke L-2-HGDH enzym zou theoretisch hersteld kunnen worden door het verhogen van de fysiologische FAD concentratie. Verhoogde FAD concentraties lieten *in vitro* een verhoogde katalytische activiteit zien van L-2-HGDH in gezuiverde enzymextracten uit rattenlever [4]. Van de elf onderzochte L-2-HGDH deficiënte lymfoblasten cellijnen was er bij één cellijn residuele activiteit meetbaar (20% van de gemiddelde controle activiteit) (**Chapter 2**). Het herstellen van de enzymactiviteit met het verhogen van de fysiologische FAD concentraties is een mogelijke therapeutische strategie dat verder onderzocht dient te worden.

Vierentwintig D-2-HGA patiënten zijn beschreven met gemeenschappelijke genetische, enzymatische en biochemische eigenschappen en deze patiënten onderscheiden zich als groep in een cohort van in totaal vijftig D-2-HGA patiënten (**Chapter 3**). Deze patiënten hebben mutaties in het *D2HGDH* gen, hebben een verminderde D-2-hydroxyglutaraat dehydrogenase (D-2-HGDH) activiteit en zijn vervolgens aangeduid als D-2-HGA type I, een autosomaal recessieve vorm van D-2-HGA. Deze patiënten hebben significant lagere D-2-hydroxyglutaraat (D-2-HG) concentraties in urine, plasma en liquor vergeleken met D-2-HGA patiënten die geen mutaties hebben in het *D2HGDH* gen.

In D-2-HGA is een nieuw metabool defect ontdekt met de identificering van *de novo* heterozygote germline mutaties in *isocitraat dehydrogenase 2* (*IDH2*) (**Chapter 4**). Deze patiënten hebben geen mutaties in *D2HGDH*, hebben een normale D-2-HGDH enzymactiviteit en zijn vervolgens aangeduid als D-2-HGA type II, een autosomaal dominante vorm van D-2-HGA. De mutatie in IDH2 resulteert in het ontstaan van een nieuwe functie (gain-of-function) van het enzym, namelijk de mogelijkheid om 2-KG om te zetten naar D-2-HG. In D-2-HGA type II wordt een significant hogere fysiologische D-2-HG concentratie gevonden dan in D-2-HGA type I. In enkele ongerelateerde D-2-HGA patiënten zijn geen mutaties gevonden in *D2HGDH* en *IDH2*, het defect in deze patiënten blijft dus onbekend.

Een enzymmethode toegepast in lymfoblast-lysaten van patiënten bevestigde de aanwezigheid van de IDH2-mutant gain-of-function activiteit in D-2-HGA type II (**Chapter 5**). Met LC-MS/MS is de omzetting van stabiel gelabeld 2-KG naar D-2-HG gedetecteerd. De D-2-HG productie was acht keer hoger in D-2-HGA type II vergeleken met controles en D-2-HGA type I. Hoewel D-2-HGDH normaal functioneert in D-2-HGA type II patiënten, is de capaciteit van D-2-HGDH blijkbaar ontoereikend om het door de IDH2-mutant gevormde D-2-HG volledig te verwerken. De IDH2-mutant enzymmethode is toegepast voor het verkennen van therapeutische mogelijkheden. Een screening is uitgevoerd met van nature in de mens aanwezige metabolieten die chemisch gelijkenis vertonen met 2-KG, D-2-HG en isocitraat. Oxaloacetaat bleek de meest krachtige competitieve remmer te zijn om D-2-HG productie te minderen.

De erkenning van D-2-HGA type I en D-2-HGA type II als afzonderlijke stofwisselingsziekten werd aangevuld met de beschrijving van het fenotype (**Chapter 6**). Klinische data zijn verzameld met behulp van een enquête. D-2-HGA type I en type II delen het biochemische kenmerk van de D-2-HG accumulatie in lichaamsvloeistoffen, dat waarschijnlijk correleert met de in beide stoornissen frequent waargenomen symptomen als *ontwikkelingsvertraging*, *hypotonie* en *epilepsie*. Cardiomyopathie wordt voornamelijk gevonden in D-2-HGA type II en is vermoedelijk gerelateerd aan verlaagde (mitochondriale) NADPH en 2-KG niveaus.

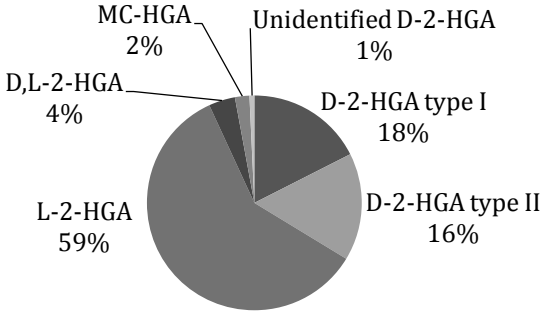
Het ziekte veroorzakende IDH gain-of-function mechanisme in D-2-HGA type II wordt vaak gevonden in neoplastische aandoeningen [8,9]. Totnogtoe is er bij patiënten aangedaan met D-2-HGA type I of type II geen kanker geconstateerd, waardoor op dit moment er geen ondersteunende data is voor de door Dang et al. voorgestelde rol van D-2-HG als “onco-metaboliet”. In L-2-HGA is daarentegen een verhoogd risico op hersentumoren gevonden. Dit suggereert dat L-2-HG directe of indirecte carcinogene eigenschappen heeft. De ontdekking van het identieke mechanisme van D-2-HG accumulatie in D-2-HGA type II en kanker verbindt deze ziekten met elkaar, en dit kan tot nieuwe inzichten leiden betreffende de pathofysiologie.

D-2-HGA en L-2-HGA vertonen een zeer verschillend klinische beeld, wijzend naar verschillende pathofysiologische invloeden van D-2-HG en L-2-HG accumulatie in de mens. Zodra de momenteel nog slecht begrepen pathofysiologische mechanismen van deze aandoeningen beter in kaart gebracht zijn zal dit de ontwikkeling van therapeutische strategieën kunnen bevorderen.

Het (genetische) defect in D,L-2-HGA is nog onbekend, maar de overeenkomende klinische en metabole bevindingen in vijf casussen suggereren de aanwezigheid van één ziekteverwekkend mechanisme voor deze ernstige neonatale epileptische encefalopathie. Exome sequencing wordt beschouwd als een potentiële strategie voor verder onderzoek om het defect op te sporen in deze patiënten.

Eind conclusies

Dit *Proefschrift* beschrijft het bestaan van ten minste twee D-2-HGA varianten: D-2-HGA type I en D-2-HGA type II. Vier verschillende neurometabole aandoeningen omvatten de meerderheid van de gediagnosticeerde 2-HGA patiënten: L-2-HGA, D-2-HGA type I, D-2-HGA type II en D,L-2-HGA (figuur 1). Recentelijk zijn patiënten beschreven met IDH1 gain-of-function mutaties die aangedaan zijn met gecombineerde Metaphyseal Chondromatosis en D-2-HGA (MC-HGA) [10], maar een klein aantal patiënten met diverse fenotypen hebben een (nochtans) onbekende oorzaak van D-2-HG accumulatie. Dit *Proefschrift* beschrijft de nader verworven kennis betreffende de metabole, enzymatische, genetische en klinische kenmerken van D-2-HGA type I en L-2-HGA, alsmede de ontdekking en eerste beschrijving van D-2-HGA type II. De initiëring van de eerste *in vitro* verkenningen omtrent de ontwikkeling van therapeutische interventies in D-2-HGA type II maken ook deel uit van dit *Proefschrift*. Het doorgronden van de pathofysiologische mechanismen in 2-HGA zou kunnen helpen bij het vinden van een goede therapie voor deze aandoeningen of voor de bescherming van de mogelijk schadelijke effecten van D-2-HG en L-2-HG accumulatie.



Figuur 1: Totale 2-hydroxyglutaarzuuracidurie verdeling (n=276)

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Kataññutā (Dankbaarheid)

Mijn reizen in het Verre Oosten hebben mij in contact gebracht met Boeddhisten, voor wie dankbaarheid, *kataññutā*, een onafscheidelijk onderdeel is van het leven. Hier probeer ik de betekenis van *kataññutā* te beschrijven, zodat jullie hopelijk de dankbaarheid kunnen voelen die ik heb voor jullie allen die mij ondersteund hebben gedurende de tijd die ik besteed heb aan het maken van dit *Proefschrift*, alsook aan iedereen die met mij het levenspad bewandelen. Daarvoor heb ik een "samenvatting en vrije interpretatie" gemaakt van het werk GRATITUDE gepubliceerd door Mahinda Wijesinghe* dat de betekenis van *kataññutā* uiteenzet.

> **"In de wereld zijn twee personen zeldzaam. Welke twee? In de eerste plaats, diegene die zich onzelfzuchtig in dienst stelt voor de hulp aan anderen (*pubbakari*). En als tweede, diegene die dankbaar is (*kataññu*) en wederkerig helpt (*katavedi*)."**
>
> **- Boeddha -**

Deze woorden van Boeddha, vertaald uit de Theravada-Boeddhistische taal Pāli, verwijst naar *kataññutā*, een definitie voor "het weten of erkennen van een handeling". Met andere woorden, de erkenning van een handeling gedaan voor iemand of voor iemands belang, vaak begeleid met een gevoel om dankbaarheid te willen uitdrukken. Volgens de Boeddhistische traditie zijn de drie belangrijkste vormen waar dankbaarheid zich op richt: In de eerste plaats onze ouders, gevolgd door onze leermeesters, en als derde onze "spirituele" vrienden.

1 Onze ouders hebben ons het leven gegeven, zij hebben ons een menselijk lichaam gegeven. Onze ouders geven ons niet alleen een menselijk lichaam, maar zij voeden ons op zo goed als ze kunnen. Zij stellen ons in staat om te overleven, zij leren ons te spreken en zij onderwijzen ons. Dit is de basis van de meeste dingen die we later leren. Daarom tonen wij dankbaarheid aan onze ouders.

2 Met leermeesters wordt bedoeld degenen van wie wij ons onderwijs krijgen, niet alleen academisch, maar ook cultureel. In dit opzicht, hebben onze schoolmeesters uiteraard een belangrijke plaats, maar ook onze leidinggevenden en collega's. Heel weinig van wat we weten is een resultaat van onze eigen inspanningen. Vrijwel alles wat we weten is ons geleerd op een of andere manier. Bijvoorbeeld als we denken aan onze wetenschappelijke kennis. Wetenschappers voeren experimenten uit en vinden nieuwe feiten, die niemand anders ooit heeft ontdekt. Hierdoor worden stukjes nieuwe kennis toegevoegd aan de wetenschappelijke kennis. Echter, dit is alleen

*(http://www.beyondthenet.net/thedway/nyanadassana-ebooks/gratitude%20in%20the%20buddhas%20teaching.pdf)

mogelijk omdat anderen veel werk hebben gedaan op dit gebied, voor ons, waarop nieuwe kennis en ideeën gevormd zijn. We hebben geprofiteerd van hun inspanningen. Onze kennis, zo kunnen we stellen, is gebaseerd op een echo daarvan. Bovendien hebben we niet alleen onze kennis vergaard in een puur intellectuele zin, maar ook hebben wij ons gemeenschappelijk cultureel erfgoed ontvangen van schrijvers, arbeiders, schilders, uitvinders, ambachtslieden, beeldhouwers, politici, componisten, filosofen en van iedereen die ons iets heeft geleerd. Daarom tonen wij dankbaarheid aan onze leermeesters.

3 De derde en voornaamste personen waar wij onze dankbaarheid aan betuigen zijn onze "geestelijke" vrienden. In het Sanskriet worden de woorden *kalyāna mitra* vaak aangeduid als "spirituele" vriend. *Mitra* komt van het woord *maitri* of *metta* in Pāli, wat sterke, onzelfzuchtige, actieve liefde betekent. In de Boeddhistische traditie wordt het scherp onderscheidden van *prema*, in de zin van de seksuele liefde of gehechtheid. Een m*itra* of vriend betekent iemand die een sterke onzelfzuchtige liefde en verbondenheid voelt voor de ander. *Kalyāna* betekent mooi, charmant, en als tweede betekent dit gunstig, behulpzaam, moreel goed. Dus, *kalyāna mitra* heeft een veel rijkere betekenis dan alleen "spirituele" vriend. Wie zijn onze *kalyāna mitras*? Zij zijn al diegenen die meer spiritueel ervaren en ontwikkeld zijn dan wij. De Boeddha's zijn (natuurlijk) onze spirituele vrienden, in het bijzonder Shakyamuni Boeddha, de ontdekker en leermeester van de Dhamma (de Universele Wet van de Natuur in het Boeddhisme). In mijn vrije interpretatie, *kalyāna mitras* zijn familie, vrienden, collega's en alle andere personen die wij in het leven ontmoeten en met wie we echt contact maken en ons de mentale, fysieke en emotionele aspecten van het leven leren ervaren. Daarom tonen wij dankbaarheid aan onze "spirituele" vrienden.

Kataññutā

Mijn ouders hebben mij leven gegeven, mijn leermeesters hebben mij onderwijs, kennis en cultuur gegeven, mijn "spirituele" vrienden hebben mij leiding gegeven in het leven (*pubbakari*).

Dit gezegd hebbende, erken ik met dankbaarheid vanuit het diepste van mijn hart alle ondersteuning, liefde, interesse, hulp, conversaties, ideeën, plezier, vriendschap en al het andere dat ik van jullie heb mogen ontvangen in de afgelopen vier jaar, en zeker ook de dertig jaar die daar aan vooraf zijn gegaan (*kataññu*). Ik hoop dat ik jullie wederkerig kan en zal helpen (*katavedi*).

Als we dankbaar zijn, zijn we verzekerd van geluk.

Kataññutā (Gratitude)

My travels in the Far East brought me in contact with Buddhists, for who gratitude, *kataññutā*, is an inseparable aspect of life. Here, I'll try to depict the meaning of *kataññutā*, so that you hopefully can feel the gratitude I have for you all who supported me during the time I spent to prepare this *Thesis*, as well as to all of you who are with me on the road of life. I made a "summary and free interpretation" of the work GRATITUDE published by Mahinda Wijesinghe* which describes the meaning of *kataññutā*.

> **"There are two persons who are rare in the world. Which two? First, the one who volunteers to help others selflessly (*pubbakari*). And second, the one who is grateful (*kataññu*) and helps in return (*katavedi*)"**
>
> **- Buddha -**

This phrase of Buddha, translated from the Theravada-Buddhism language Pāli, refers to *kataññutā*, which means "knowing or recognizing what has been done". That is to say, recognition for what has been done to one or for one's benefit, often accompanied with a feeling to express one's gratitude. In traditional Buddhism, there are three principal forms of gratitude: In the first place are our parents, next our teachers, and in the third place our "spiritual" friends.

1 Our parents have given us life, they've given us a human body. Not only do our parents give us a human body, they bring us up, well as best they can. They enable us to survive, they teach us to speak and they educate us. This is the basis of most of the things that we subsequently learn. Therefore, we are grateful to our parents.

2 By teachers is meant those from whom we derive our education, not only academically, but also culturally. In this regard, our schoolteachers have obviously an important place, but also our supervisors and peers. We have found out very little of what we know for ourselves, as a result of our own efforts. Practically everything that we know has been taught us in one way or another. For example if we think of our knowledge of science. Scientists perform experiments and find new facts, which no one else had discovered. By doing this, pieces of new knowledge are added to the knowledge of science. However, this was only possible since many others have done much work in this field before, for us, on which new knowledge and ideas are formed. We have benefited from their efforts. Our knowledge, we may say, is based on an echo

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of theirs. Additionally, we learned not only from them in a purely intellectual sense, but also our collective cultural heritage received from writers, laborers, painters, inventors, craftsmen, sculptors, politicians, composers, philosophers and anybody who tough us something. For that, we are grateful to our teachers.

3 The third principal form of our gratitude, are the "spiritual" friends. The Sanskrit words *kalyāna mitra* is often referred as "spiritual" friend. *Mitra* comes from the word *maitri* or *metta* in Pāli, which means the strong, unselfish, active love. It is sharply distinguished in Buddhist tradition from *prema*, in the sense of sexual love or attachment. A *mitra* or friend in this sense therefore means one who feels a strong unselfish active love towards one. *Kalyāna* means beautiful, charming, and secondly it means auspicious, helpful, morally good. Thus, *kalyāna mitra* has a much richer connotation than "spiritual" friend. Who are *kalyāna mitra*? They are all those who are more spiritually experienced and developed than we are. The Buddhas (of course) are our spiritual friends, especially Shakyamuni Buddha, who discovered and taught the Dhamma (The Universal Law of Nature in Buddhism). In my free interpretation, *kalyāna mitras* are family, friends, colleagues and all other persons who we encounter in life with who we make real connection and teach us how to experience the mental, physical and emotional aspects of life. Consequently, we are grateful to our "spiritual" friends.

Kataññutā

My parents have given me life, my teachers have given me education, knowledge and culture, my "spiritual" friends have given me guidance in life (*pubbakari)*.

Having said this, I acknowledge with utmost gratitude the support, love, interests, help, conversations, ideas, pleasure, companionship and much more I received from all of you in the last four years, as well as the thirty years prior to this period (*kataññu*). I hope I can and will help you in return (*katavedi*).

If we are grateful, we are sure to be happy.

Cover description

Lake Keitele (1905) by Akseli Gallen-Kallela

Akseli Gallen-Kallela (26 April 1865, Pori - 7 March 1931) was a leading figure in modern Finnish painting and decorative arts, who studied painting in Helsinki and Paris. On returning to Finland, he became fascinated with the 'Kalevala' epic, a compilation of ancient poetry celebrating the mythic origins of Finland, on which he decided to base his art. By the mid-1890s Gallen-Kallela began to incorporate symbolist motifs in his work. His landscape paintings of about 1900 show the influence of both Gauguin and Monet. Gallen-Kallela first worked at Lake Keitele, north of Helsinki, in summer 1904. The above landscape is his third and most elaborate depiction of the lake.

In October 2011, during the final period of my PhD-period, I visited The National Gallery in London for only half an hour during a city trip. I was immediately struck by this painting due to its very realistic appearance from a large distance when I entered the exhibition hall, but up close it appeared to be a real impressionistic painting. I recognized the symbolism of the island in the water as isolated (2-HGA) patients who lack proper therapy and are so limited in numbers to be able to share their difficulties amongst equals. The reflection of the island symbolizes for me the mirror molecules (the chiral molecules D-2-HG and L-2-HG, see page 54 Figure 2) which I studied for four years in these patients. Therefore, I chose to use this painting for my thesis-cover.

About the author

Martijn Kranendijk was born 2nd of May, 1977 in Delft, The Netherlands. In 1994 he graduated secondary education (HAVO) at Willem van Oranje college in Waalwijk, after which he started the study Analytical Chemistry at Hogeschool Arnhem. After one year he switched to General Chemistry at Rijkshogeschool IJsselland in Deventer and received his Bachelor/Engineer degree (BSc./Ing.) in 1999. During this study a two month minor internship was performed at the Nottingham Trent University, England: An educational website was developed for the "Organometallic" class. A nine month major internship was performed at Department of Molecular Spectroscopy, BU Fibers (currently Teijin Twaron), Akzo Nobel in Arnhem, The Netherlands for which the Biorad GC-IR interface Tracer was installed and implemented for polymeric research and troubleshooting. Subsequently, he was employed for nine months as an Analytical Chemist IR, NMR and GC-IR-MS.

After three month backpacking in the USA, Martijn was appointed as a Pharmaceutical Scientist Analytics at Department of Pharmaceutics, NV Organon (currently MSD) in Oss, The Netherlands in 2000. Many analytical techniques (*e.g.* HPLC, LC-MS, physical tests) were applied for the development of inventive early stage medicines. In 2001 he started the study Chemistry in part-time at the University of Utrecht, The Netherlands and received his Master degree/Doctorandus (MSc./Drs.) with *honours* (*geslaagd met genoegen*) in 2006. His major internship was performed at the department Martijn was employed with the research topic: Evaluation of the sensitivity of miniaturized LC-MS for pharmaceutical analysis.

In 2006, Martijn resigned working for NV Organon to travel for eight months together with his spouse through Russia, Mongolia, China, Tibet, Nepal, India/Asia and finalized his journey in Australia where he started working for six months as an Analytical Scientist at Department of Analytical Development, Acrux Ltd. in Melbourne in 2007. Novel transdermal spray technologies were developed containing generic drug substances combined with enhancers.

After returning to The Netherlands, Martijn was invited to perform research into inborn errors of metabolism at the Metabolic Unit of Prof. dr. C. Jakobs, Department of Clinical Chemistry, VU University Medical Center, Amsterdam in 2008. He was appointed for the PhD thesis on 2-hydroxyglutaric acidurias, of which the results are described in this *Thesis*. He received the *Wadman en Van Gennip price 2010* for Best Young Researcher in the field of Inherited Metabolic Diseases awarded by the Dutch Inherited Metabolic Diseases Society (ESN). The *Science Award Clinical Chemistry 2010* awarded by the Dutch Society of Clinical Chemistry and Laboratory Healthcare (NVKC) was appointed to the publication "M. Kranendijk et al. *IDH2 mutations in patients with D-2-hydroxyglutaric aciduria*, Science 2010".

Martijn is married to Marjolein Kranendijk-Eilander and they live together with their son Kasper in Utrecht.

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